Controlled Pollination with Sorted Reduced and Unreduced Pollen Grains Reveals Unreduced Embryo Sac Formation in *Diospyros kaki* Thunb. ‘Fujiwaragosho’

Ayumi Yamada and Ryutaro Tao*

Laboratory of Pomology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606–8502, Japan

This study investigated ploidy levels of endosperm and embryos in seeds of the hexaploid (2n=6x=90) *Diospyros kaki* ‘Fujiwaragosho’ to determine the possible origin of unusual ploidy levels observed in seedlings of this cultivar. Female flowers of ‘Fujiwaragosho’ were pollinated with sorted reduced (3x) and unreduced (6x) pollen grains of ‘Zenjimaru’ in 2004 and 2005. Embryo rescue culture was conducted with normally developing seeds that were excised from immature fruit harvested in August. Ploidy levels of the endosperm and plantlets from the embryos in the seeds were determined by flow cytometry. In some cases, chromosomes of root tip cells of the plantlets were also counted to determine ploidy levels of the embryos. It appeared that seeds obtained with reduced pollen pollination contained a normal hexaploid embryo and nonaploid (9x) endosperm, while those obtained with unreduced pollen pollination mostly contained a dodecaploid (12x) embryo and octadecaploid (18x) endosperm. These results indicated unreduced embryo sac formation in ‘Fujiwaragosho’. We discuss the possible cause of the occurrence of only nonaploid and octadecaploid endosperm, respectively, with reduced and unreduced pollen pollinations.

Key Words: *Diospyros kaki*, flow cytometry, sexual polyploidization, unreduced female gametes.

---

Introduction

Kaki or Japanese persimmon (*Diospyros kaki*) is a polyploid fruit tree species that has been cultivated for over ten centuries in Japan. Although most commercial kaki cultivars grown in Japan are hexaploid (2n=6x, x=15) (Namikawa and Higashi, 1928), there are a few nonaploid (intra-specific triploid) seedless cultivars such as ‘Hiratanenashi’ (2n=9x) (Zhuang et al., 1990). ‘Hiratanenashi’ shows characters commonly observed in triploids (Sanford, 1983) such as vigorous growth, seedlessness, and enlarged cell size, which may account for its high yield and high fruit quality. Due to these characters, ‘Hiratanenashi’ and its sport cultivars account for nearly 35% of kaki production in Japan. We propose, therefore, that nonaploid kaki breeding may be a promising way to obtain new cultivars with commercially desirable characteristics.

We have developed several polyploidy breeding methods for kaki, such as endosperm culture, colchicine treatment to protoplast, protoplast fusion, and sexual polyploidization (Sugiura et al., 2000; Tamura et al., 1995, 1996; Tao et al., 1997). Among them, sexual polyploidization using unreduced gametes (2n) seems to be the most promising way because introgression of new genetic traits to polyploids is possible and the method is relatively simple. Unreduced gametes were reported to be produced in many plant species including potato (Mendiburu and Peloquin, 1977), blueberry (Lyrene and Ballington, 1986), and alfalfa (Tavoletti et al., 1991), and thought to be the principal origin of natural polyploids (Ramanna and Jacobsen, 2003). Unreduced male gametes or unreduced pollen is also produced in several kaki cultivars, and Sugiura et al. (2000) successfully obtained nonaploids by pollinating hexaploid cultivars with unreduced pollen. With regard to unreduced female gametes, we found that a cultivar, ‘Fujiwaragosho’, could produce unreduced eggs (Tao et al., 2003), because it specifically produces polyploid seedlings with various ploidy levels when pollinated.
with hexaploid kaki cultivars (Yamada and Tao, 2006). However, the origin of the polyploid seedlings of ‘Fujiwaragosho’ is yet to be clarified. This study was conducted to determine if unreduced female gametes are involved in the formation of ‘Fujiwaragosho’ seedlings with various ploidy levels. We pollinated ‘Fujiwaragosho’ with sorted reduced and unreduced pollen grains and investigated the ploidy levels of endosperm and embryos of the resulting seeds. Based on their ploidy levels, we discuss the possible origin of ‘Fujiwaragosho’ seedlings with unusual ploidy levels.

Materials and Methods

Plant materials

Experiments were conducted in 2004–2005 at the experimental farm of Kyoto University, Japan. Two hexaploid monoecious kaki cultivars, ‘Fujiwaragosho’ and ‘Zenjimaru’, were used in this study. ‘Zenjimaru’ was used as a pollen donor because it bears many male flowers which contain much pollen. Furthermore, a previous study showed that the proportion of unreduced pollen grains in ‘Zenjimaru’ was relatively high, with about 5% of the total pollen grains being unreduced (Sugiura et al., 2000). Pollen grains of ‘Zenjimaru’ were collected from male flowers as described by Sugiura et al. (2000), and stored at 4°C or −20°C with a silica gel desiccant until use.

Pollination with either sorted reduced or unreduced pollen and embryo culture

Female flowers of ‘Fujiwaragosho’ were bagged about 1 week before flowering and pollinated with sorted reduced and unreduced ‘Zenjimaru’ pollen grains separately. Reduced and unreduced pollen grains were sorted according to Sugiura et al. (2000). In short, an aliquot of ‘Zenjimaru’ pollen was hydrated in cell and protoplast washing (CPW) solution with 0.9 M mannitol (Draper et al., 1988) for 2 h with agitation. After hydration, reduced and unreduced pollen grains were sorted using 62 µm nylon mesh. Sorted pollen grains were immediately resuspended in 15% sucrose solution and used for pollination. In 2005, pollen grains just hydrated with CPW solution but not sorted, which were supposed to contain both reduced and unreduced ones, were also used for pollination. Female flowers were left bagged at least until 7 days after pollination to prevent contamination with other pollen.

Premature fruit was collected in mid-August, about 80 days after the end of flowering. Seeds developing normally with a longitudinal length of approx. 15 mm were taken from the fruit and embryos were cultured aseptically, as described by Yamada and Tao (2006). After excision of the embryos, the endosperm of seeds was subjected to flow cytometric analysis to determine ploidy levels, as described before (Yamada and Tao, 2006).

Determination of ploidy levels

After several sub-cultures, ploidy levels of the seedlings germinated in vitro from the embryos were determined with a flow cytometer (FACSCalibur; Becton-Dickinson, NJ, USA) as described by Sugiura et al. (2000). A hexaploid cultivar, ‘Jiro’, was used as a reference standard (Tamura et al., 1998). Ploidy levels of the endosperm in seeds used for embryo culture were also determined using the Ploidy Analyzer (PA; Partec, Münster, Germany) following the manufacturer’s instructions. An aliquot of endosperm was immersed in Nuclei Extraction Buffer (Partec) and chopped with a razor blade to release nuclei. The suspension was incubated for 5 min and then passed through a 20-µm nylon mesh to remove debris. The nuclei in the filtrate were stained with Staining Buffer (Partec) containing 4,6-diamidino-2-phenylindole (DAPI). Relative nuclear DNA contents of the nuclei were determined with the Ploidy Analyzer using ‘Jiro’ leaf nuclei as a reference standard. Furthermore, if necessary, chromosomes of seedlings were also counted using root tips obtained from plantlets, as described by Choi et al. (2002, 2003).

Results and Discussion

The percentages of fruit set in ‘Fujiwaragosho’ in mid-August ranged from 17% to 45%. Two-way analysis of variance showed that neither year nor pollen type (reduced/unreduced) had a significant effect (α = 0.05) on the fruit set (Table 1). As for non-sorted pollen pollination, fruit set was not significantly different (α = 0.05) among the three pollen types in 2005 (one-way analysis of variance). When ‘Fujiwaragosho’ was

Table 1. Fruit set and seed formation after pollination with different pollen types in a 2-year experiment.

| Pollen type          | Year | Number of fruit collected (Number of seeds per fruit) |
|----------------------|------|-------------------------------------------------------|
| Reduced pollen (n)   | 2004 | 20(45)                                                |
|                      | 2005 | 18(26)                                                |
| Unreduced pollen (2n)| 2004 | 37(31)                                                |
|                      | 2005 | 23(26)                                                |
| Non-sorted pollen (n+2n) | 2005 | 12(17)                                                |

 Data were collected in mid-to-late August when almost 80 days had elapsed from flowering.

 Total numbers of fully-developed seeds with visible embryos obtained by respective treatments.
pollinated with dry kaki pollen in the 3 years from 2002 to 2004, the percentages of fruit set were higher (65–82%; Yamada and Tao, 2006) than the results obtained in this study. Thus, it is possible that the hydration and sorting process used in this study might have lowered the pollen germination rate. The number of normally-developing seeds per fruit was similar irrespective of pollinated pollen type and year (Table 1). Two-way analysis of variance showed that neither year nor pollen type (reduced/unreduced) had a significant effect (α = 0.05) on the number of normally-developing seeds per fruit. One-way analysis of variance showed that the number of normally-developing seeds per fruit was not significantly different (α = 0.05) among the three pollen types including non-sorted pollen in 2005. Many fruit contained 0, 1, or 2 normally-developing seeds, and the largest number of normally-developing seeds per fruit was 6. Seed size and embryo developmental stage were also similar among the pollinated pollen types. Seeds with fully-developed endosperm but no visible embryo were obtained from fruit pollination with all 3 pollen types (data not shown).

Ploidy levels of endosperm that were determined by the Ploidy Analyzer are shown in Table 2. Since no peak of the relative nuclear DNA content of endosperm was detected in some seeds, we could not determine their endosperm ploidy level. Except for those seeds with undetermined ploidy levels, all seeds obtained by pollination with reduced pollen contained normal nonaploid endosperm (3n = 9x), supposedly derived from the fusion of 2 triploid polar nuclei and 1 triploid sperm cell. In 2004, seeds from the cross with unreduced pollen contained either nonaploid or octadecaploid endosperm (3n = 18x). In 2005, however, all endosperm was octadecaploid. When pollinated with non-sorted pollen, seeds contained nonaploid or octadecaploid endosperm. The possible origin of octadecaploid endosperm was the fusion of 2 hexaploid polar nuclei and 1 hexaploid sperm cell, which is well correlated with the simultaneous formation of dodecaploid embryos.

Although most embryos cultured in vitro germinated and developed vigorously, 5 embryos obtained by pollination with unreduced pollen in 2004 showed retarded growth and died after several subcultures. Ploidy levels of seedlings were determined by flow cytometric analysis and in some cases, chromosome counting was also conducted to confirm ploidy estimates (Figs. 1 and 2). All seedlings from the cross with reduced pollen were shown to be hexaploid (Table 2). Consequently, all seeds obtained by the cross with reduced pollen (except for the one in which we could not determine the ploidy level of endosperm) contained...
A. Yamada and R. Tao

nonaploid endosperm and hexaploid embryos, supposedly derived from the fertilization of a reduced embryo sac with reduced pollen nuclei. When we pollinated ‘Fujiwaragosho’ with dry pollen of hexaploid kaki cultivars ‘Okugosho’ and ‘Shogatsu’ in 2002 to 2004, seeds containing polyploid embryos with higher ploidy levels than hexaploid (mostly nonaploid with some dodecaploid) and nonaploid endosperm were obtained at 6% to 80% (Yamada and Tao, 2006). Since the unreduced pollen ratios of pollen grains pollinated were very low (about 0.2% to 1%), these seeds seemed to be derived from fertilization with reduced pollen. Thus, if we had pollinated more flowers with reduced pollen in this study, polyploid seedlings should also have been obtained.

Flow cytometric analysis indicated that most seedlings from the cross with unreduced pollen (72/79) were putative dodecaploid (2n = 12x) (Table 2). In 2004, we also obtained 6 hexaploid and 1 nonaploid seedling. Therefore, seeds obtained by pollination with unreduced pollen were classified into 3 types according to the ploidy levels of the endosperm and embryo: 1) octadecaploid endosperm and dodecaploid embryo, 2) octadecaploid endosperm and nonaploid embryo, and 3) nonaploid endosperm and hexaploid embryo. Most seeds obtained were classified into the first type, which were supposed to be derived from fertilization of an embryo sac containing 2 hexaploid polar nuclei and a hexaploid egg with hexaploid pollen. These embryo sacs might have been derived from the unreduced megaspore mother cells, which were formed by the failure of meiosis. Second type seeds were supposed to be derived from fertilization with unreduced pollen, because all seeds from the cross with reduced pollen contained nonaploid endosperm. An embryo sac with 2 hexaploid polar nuclei and a triploid egg might have been formed. Since the third type seeds contained a hexaploid embryo, they may have been derived from pollination with reduced pollen contaminated with unreduced pollen.

Seeds from the cross with non-sorted pollen contained hexaploid, nonaploid, or dodecaploid embryos (Table 2). Considering the ploidy level of endosperm, seeds that contained nonaploid embryos seemed to be derived from fertilization with reduced pollen, while those that contained dodecaploid embryos seemed to be derived from fertilization with unreduced pollen. The origin of the former seeds might be an embryo sac with triploid polar nuclei and a hexaploid egg.

In this study, we pollinated ‘Fujiwaragosho’ with both reduced (3x) and unreduced (6x) pollen grains of a hexaploid kaki cultivar. Although normally-developing seeds obtained from the cross with reduced pollen contained nonaploid endosperm, those from the cross with unreduced pollen contained octadecaploid endosperm. The endosperm is likely to play an important role during seed development irrespective of its persistence in the mature seed (Berger, 1999). Many examples suggested that endosperm failure is the primary cause of seed abortion following interploidy crosses (Scott et al., 1998). Failure of seed development following interploidy crosses is also observed in kaki. When Sugiura et al. (2000) crossed a hexaploid kaki cultivar with unreduced hexaploid pollen, nonaploids were obtained from under-developed seeds with no endosperm and very small globular embryo. Lin (1984) used a maize indeterminate gametophyte (ig) mutant and showed that a ratio of 2 maternal genomes (2m) to 1 paternal genome (1p) is the critical factor for normal endosperm. ‘Fujiwaragosho’ was supposed to produce embryo sacs containing polar nuclei with various ploidy levels, including normal triploid and abnormal hexaploid. Among various embryo sac types, only those containing 2 hexaploid polar nuclei could develop into seeds when pollinated with hexaploid unreduced pollen, keeping the normal 2m:1p genomic ratio in endosperm. Since there was no significant difference in the numbers of normally-developing seeds per fruit between the cross with reduced and unreduced pollen, embryo sacs with hexaploid polar nuclei and with triploid polar nuclei could be formed at a similar frequency.

Table 2. Ploidy levels of endosperm and seedlings after pollination with different pollen types in a 2-year experiment.

| Pollen type               | Year | 9x | 18x | N D |
|---------------------------|------|----|-----|-----|
| Reduced pollen (n)        | 2004 | 19 | —   | —   |
|                           | 2005 | 19 | —   | —   |
| Unreduced pollen (2n)     | 2004 | 5  | 1   | 42  |
|                           | 2005 | —  | —   | 27  |
| Non-sorted pollen (n+2n)  | 2005 | 6  | 1   | 1   |

* Ploidy levels of endosperm of seeds from which embryos were obtained.

* ND: Seeds whose ploidy level of endosperm could not be determined are included in this category.

* Ploidy levels of embryos (EM) were determined by flow cytometric analysis of seedlings germinated from the embryos.
In our previous study, 4% to 73% of normally developing seeds obtained from ‘Fujiwaragosho’ × hexaploid kaki contained a nonaploid embryo and nonaploid endosperm (Yamada and Tao, 2006). Additionally, a few seeds with a dodecaploid embryo and nonaploid endosperm were also obtained. These seeds were supposed to be from fertilization with reduced \( n \) pollen, because the endosperm was normal nonaploid. Thus, ‘Fujiwaragosho’ seemed to produce abnormal embryo sacs with normal 3x polar nuclei and abnormal 6x or 9x eggs. Figure 3 shows possible patterns of the ploidy levels of the embryo sac in ‘Fujiwaragosho’ explaining the origins of ‘Fujiwaragosho’ seeds containing embryos and endosperm with various ploidy levels obtained previously and in the present study.

It is unclear why ‘Fujiwaragosho’ produces seedlings with various ploidy levels at a high frequency through abnormal embryo sac formation. Since we found that ‘Fujiwaragosho’ trees grown in three different locations, other than Kyoto, produced 9x seedlings (unpublished results), genetic factors seem to be involved in the formation of seedlings with various ploidy levels in ‘Fujiwaragosho’. It is intriguing whether this trait of ‘Fujiwaragosho’ can be transmittable to the progeny.

In conclusion, this study showed that the hexaploid cultivar ‘Fujiwaragosho’ crossed with hexaploid unreduced pollen produced normally-developing seeds with octadecaploid endosperm at a very high frequency. Since most seeds with octadecaploid endosperm contained dodecaploid embryos, unreduced megaspore mother cells were supposed to be produced in ‘Fujiwaragosho’ at a high frequency. ‘Fujiwaragosho’ is the first possible unreduced megaspore producer in kaki and could serve as a promising female stock plant for sexual polyploidization breeding in kaki.

**Literature Cited**

Berger, F. 1999. Endosperm development. Curr. Opin. Plant Biol. 2: 28–32.

Choi, Y. A., R. Tao, K. Yonemori and A. Sugiura. 2002. Multicolor genomic *in situ* hybridization identifies parental chromosomes in somatic hybrids of *Diospyros kaki* and *D. glandulosa*. HortScience 37: 184–186.

Choi, Y. A., R. Tao, K. Yonemori and A. Sugiura. 2003. Simultaneous visualization of 5S and 45S rDNAs in
persimmon \((Diospyros kaki)\) and several wild relatives \((Diospyros spp.)\) by fluorescent \textit{in situ} hybridization (FISH) and multi-color FISH (MCFISH). J. Amer. Soc. Hort. Sci. 128: 736–740.

Draper, J., R. Scott, P. Armitage and R. Walden. 1988. Plant genetic transformation and gene expression. Blackwell Sci. Publ., Oxford.

Lin, B.-Y. 1984. Ploidy barrier to endosperm development in maize. Genetics 107: 103–115.

Lyrene, P. M. and J. R. Ballington. 1986. Wide hybridization in \textit{Vaccinium}. HortScience 21: 52–57.

Mendiburu, A. O. and S. J. Peloquin. 1977. The significance of 2N gametes in potato breeding. Theor. Appl. Genet. 49: 53–61.

Namikawa, I. and M. Higashi. 1928. On the chromosomes in \textit{Diospyros kaki} L. F. and \textit{Diospyros lotus} L. Botanical Magazine 42: 436–438.

Ramanna, M. S. and E. Jacobsen. 2003. Relevance of sexual polyploidization for crop improvement—A review. Euphytica 133: 3–18.

Sanford, J. C. 1983. Ploidy manipulations. p. 100–123. In: J. N. Moore and J. Janick (eds.). Methods in fruit breeding. Purdue University Press, West Lafayette.

Scott, R. J., M. Spielman, J. Bailey and H. G. Dickinson. 1998. Parent-of-origin effects on seed development in \textit{Arabidopsis thaliana}. Development 125: 3329–3341.

Sugiura, A., T. Ohkuma, Y. A. Choi, R. Tao and M. Tamura. 2000. Production of nonaploid \((2n=9x)\) Japanese persimmons \((Diospyros kaki)\) by pollination with unreduced \((2n=6x)\) pollen and embryo rescue culture. J. Amer. Soc. Hort. Sci. 125: 609–614.

Tamura, M., R. Tao and A. Sugiura. 1995. Regeneration of somatic hybrids from electrofused protoplasts of Japanese persimmon \((Diospyros kaki L.)\). Plant Sci. 108: 101–107.

Tamura, M., R. Tao and A. Sugiura. 1996. Production of dodecaploid plants of Japanese persimmon \((Diospyros kaki L.)\) by cochinine treatment of protoplasts. Plant Cell Rep. 15: 470–473.

Tamura, M., R. Tao, K. Yonemori, N. Utsunomiya and A. Sugiura. 1998. Ploidy level and genome size of several \textit{Diospyros} species. J. Japan. Soc. Hort. Sci. 67: 306–312.

Tao, R., K. Ozawa, M. Tamura and A. Sugiura. 1997. Dodecaploid plant regeneration from endosperm culture of persimmon \((Diospyros kaki L.)\). Acta Hort. 436: 119–128.

Tao, R., A. Yamada, T. Esumi, H. Motosugi and A. Sugiura. 2003. Ploidy variations observed in the progeny of hexaploid Japanese persimmon \((Diospyros kaki)\) ‘Fujiwaragosho’. Hort. Res. (Japan) 2: 157–160 (In Japanese with English summary).

Tavoletti, S., A. Mariani and F. Veronesi. 1991. Cytological analysis of macro- and microsporogenesis of a diploid alfalfa clone producing male and female 2n gametes. Crop Sci. 31: 1258–1263.

Yamada, A. and R. Tao. 2006. High frequency sexual polyploidisation observed in hexaploid Japanese persimmon \((Diospyros kaki)\) ‘Fujiwaragosho’. J. Hort. Sci. Biotechnol. 81: 402–408.

Zhuang, D., A. Kitajima, M. Ishida and Y. Sobajima. 1990. Chromosome number of \textit{Diospyros kaki} cultivars. J. Japan. Soc. Hort. Sci. 59: 289–297 (in Japanese with English summary).