20 Persimmon

Shinya Kanzaki[1] and Keizo Yonemori[2]

[1] Laboratory of Horticultural Science, Faculty of Agriculture, Kinki University, Nakamachi, Nara, 631-8505, Nara, Japan
e-mail: skanz@nara.kindai.ac.jp
[2] Laboratory of Pomology, Graduate School of Agriculture, Kyoto University, Sakyo-ku Kyoto 606-8502, Kyoto, Japan

20.1 Introduction

Persimmon, *Diospyros kaki* Thunb., is a deciduous fruit tree, native to the East Asia. It is believed to have originated in the mountain area of southern China and has been cultivated as an important fruit crop in China, Korea, and Japan from prehistoric times. Persimmon fruit containing high amount of vitamin C, dietary fiber, carotenoids and polyphenols (tannins) is usually consumed as a fresh or dried fruit that was one of the important nutrition sources in old times. Young fruit has been used for obtaining tannins (persimmon oil), which is of great value for industrial uses.

Persimmon is a typical oriental fruit and less known in non-Asian countries. Most part of persimmon production is from East Asia. In 2004, the global production of persimmon totaled 2,518,123 metric tones, 72.3% from China, 11.9% from Korea and 9.2% from Japan (FAO 2004). Following these main producing countries, Brazil, Italy, and Israel are producing substantial amounts, and Australia and New Zealand are producing persimmon mainly for export. Recently, remarkable expansion in persimmon production has occurred in Spain, though persimmon statistics of FAO does not include Spanish production (Llácer and Badenes 2004). Thus, persimmon is gaining popularity as a new fruit crop in the non-Asian countries in recent years.

The genus *Diospyros* L. consists of approximately 400 species, found mostly in the tropics of Asia, Africa and Central-South America (Yonemori et al. 2000). Only few species, including *D. kaki*, are native to the temperate zone. Most wild species of the genus *Diospyros* are diploid ($2n = 2x = 30$) or tetraploid ($2n = 4x = 60$), while *D. kaki* is basically a hexaploid ($2n = 6x = 90$) (Ng 1978; Tamura et al. 1998; Choi et al. 2003a, b). Some of the seedless cultivars of *D. kaki* have been reported as nonaploid ($2n = 9x = 135$) (Zhuang et al. 1990; Tamura et al. 1998). Therefore, single or several diploid and/or tetraploid species must be involved in the polyploidization of persimmon, but so far, there is no consensus as to how persimmon acquired a high chromosome number and whether it is an auto- or allo-polyploid. In an earlier study based on morphological, geographical and cytological analysis, Ng (1978) suggested a hypothesis that *D. kaki* had originated directly from *D. roxburghii* (syn. *D. glandulosa*) through polyploidy, cultivation and selection. However, phylogenetic study based on DNA variation in the special region of cpDNA (rbcL-ORF106 and trnT-trnF) indicated that *D. glandulosa* is closely related to *D. oleifera*, which is native of the temperate region of China, and may not be the direct progenitor of *D. kaki* (Yonemori et al. 1998). Recently, phylogenetic analysis using DNA sequences of ITS and *mat*K region of some *Diospyros* species revealed that *D. galandulosa* and *D. oleifera* were relatively close to *D. kaki*, but direct relationship between *D. galandulosa* and *D. kaki* has not been proved (Yonemori et al. submitted). On the other hand, close relationship between *D. kaki* and *D. lotus* was shown in both studies (Yonemori et al. 1998; Yonemori et al. in preparation). *D. lotus* is a diploid species, widely distributed in temperate Asia and consumed as fresh or dried fruits. The molecular data indicates that *D. lotus* or its ancestral species would be associated with the speciation of *D. kaki*.

It is difficult to define and characterize the sets of homologous chromosomes based on chromosome observation under a light microscope because somatic chromosomes of *Diospyros* species are too small (Tamura et al. 1998). Recently, however, fluorescent in situ hybridization (FISH) has been applied as a new useful tool for analyzing karyotypes and phylogenetic relationships of some *Diospyros* species (Choi et al. 2003a, b). When FISH using an rDNA probe was performed, four homologous chromosomes and non-

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homologous two pairs of chromosomes carrying 45S rDNA were observed (Choi et al. 2003a). The presence of non-homologous two pairs of chromosomes bearing 45S rDNA indicates that *D. kaki* might be an allohexaploid. However, four homologous chromosomes with 45S rDNA might mean that *D. kaki* may be an autoallohexaploid or at least some chromosomes are homoeologous among the different structural genomes of *D. kaki*. Thus, the genomic composition of *D. kaki* might be an allo- or autoallo-hexaploid rather than autohexaploid, although further analysis will be required to clarify the polyploid nature of persimmon. Previously, Zhuang (1990) hypothesized that *D. kaki* might be an allohexaploid, since bivalent formation occurred regularly and few multivalents were observed in the meiosis of pollen mother cell. However, the possibility of polysomic polyploidy in persimmon genome cannot be ruled out because lack of multivalent formation does not necessarily indicate a disomic polyploid in case of species with short chromosomes (Krebs and Hancock 1989; Wolf et al. 1989; Qu et al. 1998). As discussed bellow, segregation analysis of molecular markers indicated the existence of polysomic inheritance in *D. kaki* (Kanzaki et al. 2001).

The polyploidy nature in persimmon makes genetic linkage analysis difficult and, so far, no effort has been made to develop a genetic map for persimmon. Recently, we have developed molecular markers associated with the trait of natural astringency-loss in persimmon fruit and the markers are practically useful in persimmon breeding programs (Kanzaki et al. 2001). Through the analysis of the markers, a possible explanation has given about genetic nature of the trait of natural astringency-loss. In this chapter, we focus on the trait of astringency-loss and describe the possibility of polysomic inheritance in persimmon.

20.2 Nature of Natural Astringency-Loss in Persimmon Fruit and Its Inheritance

Generally, a persimmon fruit accumulates high amount of soluble tannins and tastes extremely astringent. However, some cultivars are genetically defined to lose astringency naturally on the tree as fruit development and are called as 'non-astringent persimmon' or 'sweet persimmon'. Strictly speaking, persimmon cultivars are classified into four types based on the relationship between astringency in the fruit at harvest, presence of seed, and flesh color (Hume 1914; Kajiura 1946; Yonemori et al. 2000). These four types are: 1) pollination-constant non-astringent (PCNA), 2) pollination-variant non-astringent (PVNA), 3) pollination-variant astringent (PVA), and 4) pollination-constant astringent (PCA). Among these four types, the only inborn non-astringent type is PCNA-type because PVNA-type fruits lose astringency only when they have a sufficient number of seeds. The decisive difference between PCNA and the other three types (non-PCNA-type) is the pattern of tannin accumulation in fruits (Yonemori and Matsushima 1985). PCNA-type fruits stop to accumulate tannins at the early stage of fruit growth, while non-PCNA-type accumulates tannins until the middle stage of fruit development. Therefore, PCNA-type fruits contain much less tannins than non-PCNA-type at maturity and low amount of tannins results in easy deastringency in PCNA-type fruit. In other words, PCNA-type lacks the ability to accumulate high amount of tannins in the fruits. The PCNA/non-PCNA trait is qualitatively inherited to the progenies and PCNA-type is recessive to non-PCNA-type (Ikeda et al. 1985; Yamada and Sato 2002). According to their reports, crosses among PCNA-type plants yielded only PCNA-type offspring and all F_1 hybrids between PCNA and non-PCNA-type cultivars become non-PCNA-type offspring. When these F_1 hybrids were backcrossed to PCNA-type cultivars/selections, only around 15% of PCNA-type offspring were segregated in the backcross population. Thus, it can be said that PCNA-type is a recessive mutant in which the mutation has occurred on the gene(s) controlling tannin accumulation (called *Ast*, for astringency). The low ratio of PCNA-type offspring in the backcrossed population might be caused by polyploid nature of persimmon. Assuming that persimmon is an allohexaploid (or disomic hexaploid), Ikeda et al. (1985) suggested a hypothesis that each of three structural genomes would have single *Ast* locus (*Ast1*, *Ast2*, and *Ast3*) and PCNA phenotype can be expressed only in recessive genotype with triplicate genes (*ast1 ast2 ast3*). In such a case, the expected ratio of PCNA-type offspring in the backcross population would be 12.5% if the donor parent (non-PCNA-type) was homozygous dominant genotype with the three genes. Recently, however, based on the segregation pattern of molecular markers

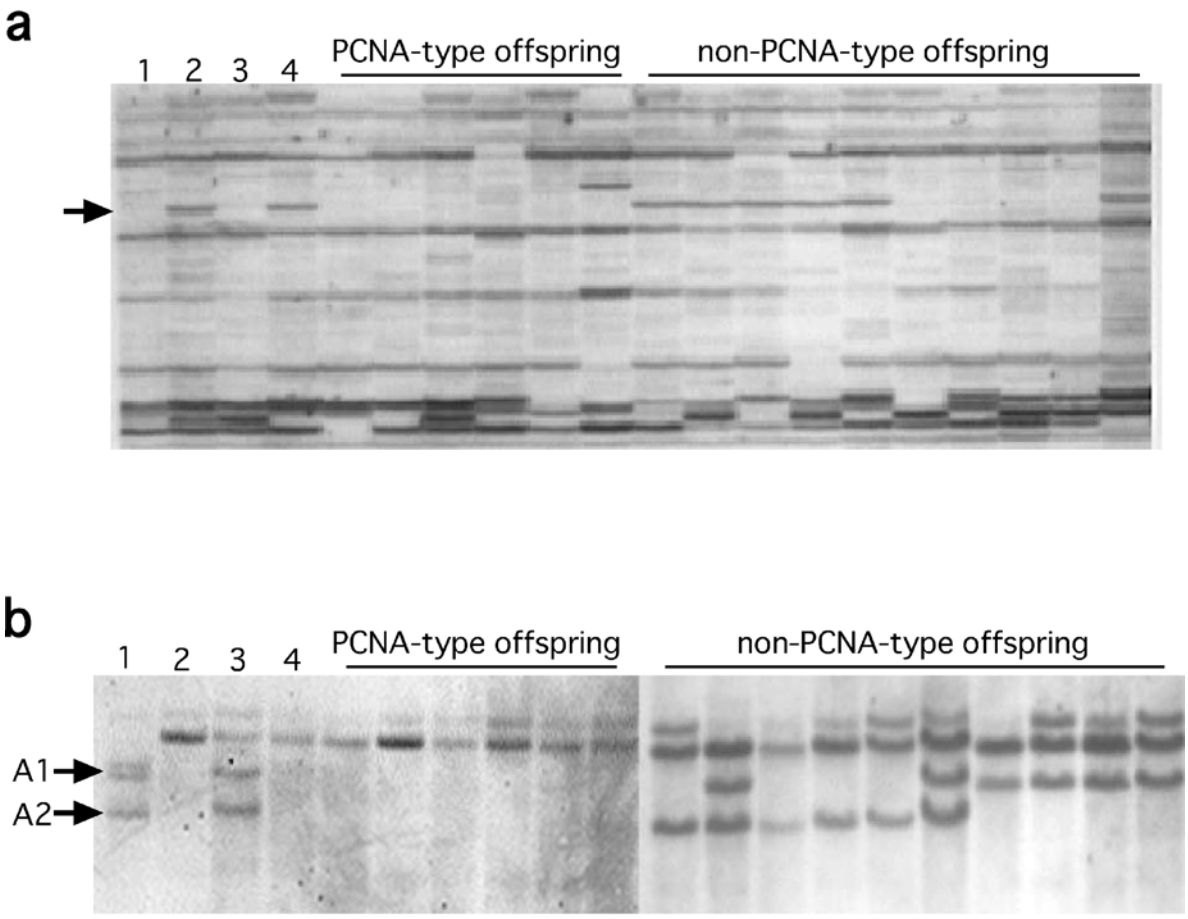

Fig. 1. **(a)** A part of AFLP fingerprints using primer combination EACC/MCTA. *Lanes*: 1, PCNA bulk; 2, non-PCNA bulk; 3, PCNA parent; 4, non-PCNA parent; following PCNA and non-PCNA offspring used for bulked segregant analysis. *Arrow* indicates the AFLP marker, EACC/MCTA-400. **(b)** RFLP analysis of genomic DNAs digested with *Hin*dIII, using EACC/MCTA-400 as a probe. *Lanes*: 1, non-PCNA donor parent; 2, PCNA parent for F_1 hybrid; 3, F_1 hybrid; 4, PCNA parent for backcross; following PCNA and non-PCNA offspring used for bulked segregant analysis. *Arrow* indicates the two RFLP markers (A1 and A2) linked to *Ast* locus

linked to the PCNA/non-PCNA trait, Kanzaki et al. (2001) suggested a new hypothesis that the mode of inheritance of *Ast* gene appeared to be polysomic rather than disomic.

20.3 Identification of Molecular Markers Liked to the PCNA/non-PCNA Trait and Polysomic Segregation of the Markers

AFLP analysis was conducted to identify molecular markers linked to the trait of natural astringency-loss in PCNA type (Kanzaki et al. 2001). A total of 128 primer combinations were used in a bulked segregant analysis and a candidate marker linked to one of the dominant alleles conferring non-PCNA trait was identified (Fig. 1a). This marker (EACC/MCTA-400) was absent in all PCNA-type offspring tested and was present in half of the non-PCNA-type offspring. When the EACC/MCTA-400 fragment was isolated and used as a probe for RFLP analysis, two polymorphic markers (A1 and A2) were detected (Fig. 1b). The segregation pattern of A2 marker in RFLP analysis was the same as that of EACC/MCTA-400 obtained by AFLP analysis. A1 marker could be detected in all A2-negative non-PCNA-type offspring and some A2-positive non-PCNA-type offspring. These results indicate that EACC/MCTA-400 and A2 markers are linked to one dominant allele and A1 marker is linked to another dominant allele. In our breeding population, all non-PCNA-type offspring could be distin-

Table 1. Segregation analysis of the RFLP markers associated with PCNA/non-PCNA trait. F_1 progeny is derived from the cross PCNA[a] × non-PCNA and backcross progeny is derived from PCNA × non-PCNA-type F_1

	Observed segregation of RFLP markers[b]	Genotype of non-PCNA parent	Expected segregation of RFLP markers	χ^2	P
F_1 progeny	8 A1 : 22 A1A2 : 10 A2				
	Disomic model	*Ast1/Ast1 Ast2/Ast2*	All A1A2	**	**
		Ast1/ast1 Ast2/ast2	1 A1: 1 A1A2: 1 A2 : 1 a	24.8	< 0.01
		Ast1/Ast2 Ast1/Ast2	1 A1: 2 A1A2 : 1 A2	0.6	0.74
	Tetrasomic model	*Ast-1/Ast-1/Ast-2/Ast-2*	1 A1 : 4 A1A2 : 1 A2	2.75	0.25
Backcross progeny	14 A1A2 : 37 A1 : 27 A2 : 23 a				
	Disomic model	*Ast1/ast1 Ast2/ast2*	1 A1A2 : 1 A1: 1 A2 : 1 a	10.8	0.013
	Tetrasomic model	*Ast-1/Ast-2/ast/ast*	1 A1A2 : 2 A1: 2 A2 : 1 a	4.39	0.22

[a] Assume that genotype of PCNA parent is *ast1/ast1 ast2/ast2* (disomic model) or *ast/ast/ast/ast* (tetrasomic model).

[b] Four RFLP patterns are represented as A1(showing only A1 marker), A2 (showing only A2 marker), A1A2 (showing both A1 and A2 markers), and a (showing neither markers).

guished from PCNA-type offspring by the presence of either RFLP marker or both. This suggests that there are two DNA fragments which are associated separately with gene(s) conferring the non-PCNA trait, and that the gene linked with each fragment is able to express the same non-PCNA trait. Contrary to the Ikeda's hypothesis (1985) that triplicate genes could be associated with the trait, our results indicated that PCNA/non-PCNA trait would be controlled by duplicate genes. Here, we suggest a possible hypothesis about the inheritance of the trait based on the segregation analysis of these RFLP markers in F_1 and backcross progenies.

Assuming that persimmon is a disomic poplyploid (allohexaploid) and homeoalleic *Ast* genes are separately associated with the trait, two RFLP markers (A1 and A2) linked to each *Ast* gene (*Ast1* and *Ast2*) should be segregated independently in a progeny. For example, if the genotype of non-PCNA-parent is homozygous for two loci (*Ast1/Ast1 Ast2/Ast2*), all F_1 hybrids between PCNA-parent (genotype: *ast1/ast1 ast2/ast2*) must show both A1 and A2 markers (Table 1). If the non-PCNA-parent has heterozygous state for both loci (*Ast1/ast1 Ast2/ast2)*, A1 and A2 bands will segregate independently and 25% of F_1 progeny should present neither A1 nor A2 markers. These assumptions, however, did not fit to the observed segregation of these markers in F_1 progeny (Table 1). It might be possible that both A1 and A2 markers are linked to homozygous dominant alleles at each locus. Assuming that *Ast1* and *Ast2* form two allele pairs at different loci (*Ast1*:*Ast2 Ast1*:*Ast2*), the expected segregation of A1 and A2 markers in F_1 progeny should be A1:A1A2:A1 = 1:2:1, and this ratio seems to fit to the observed segregation (Table 1). However, when the segregation in backcross progenies was considered, disomic model did not fit to the observed ratio (Table 1). Thus, it would not be most likely that duplicate *Ast* loci with a disomic nature control the PCNA/non-PCNA trait.

On the other hand, assuming tetrasomic inheritance of the *Ast* gene seemed to be more likely (Table 1). If persimmon is an autoallohexaploid and the PCNA/non-PCNA trait is controlled by tetra-alleic *Ast* locus in the polysomic genome, segregation of the RFLP markers linked to the locus would follow tetrasomic segregation. The observed segregation of the RFLP markers in both F_1 and backcross progenies did not deviated from the expected tetrasomic segregation (pure chromosome segregation) (Table 1) and this result suggests the polysomic nature of the locus. In addition, the expected ratio of PCNA plants in backcross progeny would be 16.7% (tetrasomic pure chromosome segregation) and this seemed to be consistent with the ratio of PCNA-type plants in the breeding population previously reported (Ikeda et al. 1985; Yamada and Sato 2002).

There exists no conclusive evidence that persimmon is an autoallohexaploid. Both of cytogeneic analysis (Choi et al. 2003a) and segregation analysis of RFLP markers (Kanzaki et al. 2001) indicates that persimmon may have four chromosomes in a homologous group. To elucidate the genomic composition, further cytogenetic studies and segregation analysis using codominant molecular markers will be required. Genetic studies on persimmon would not progress without solving this issue.

20.4 Future Scope for Persimmon Breeding

PCNA type is the most desirable for fresh consumption. Thus, to develop and release commercially attractive PCNA-type cultivars have been the main breeding objective in Japan. So far, several PCNA type cultivars have been released from the breeding programs in National Institute of Fruit Tree Science (Yonemori et al. 2000). In the Japanese breeding programs, PCNA-type cultivars/selections have been used as both parents to obtain PCNA-type offspring exclusively in the progenies. However, as crossings among PCNA-type cultivars/selections were repeated, inbreeding depression becomes a serious problem (Yamada 1993). Using non-PCNA-type cultivars/selections as a source of breeding is the better way for extending the genetic pool of breeding population, but it had been an impractical and inefficient strategy to develop PCNA-type cultivars. All F_1 hybrids between PCNA and non-PCNA type cultivars become non-PCNA type and only around 15% of PCNA-type offspring is obtainable even in backcross population. For making such a strategy more practical, marker-assisted selection using the RFLP markers linked to *Ast* locus is a useful system. We are developing an easy PCR-based selection system based on the DNA sequences of adjacent region of the RFLP markers because RFLP analysis is a relatively laborious and inconvenient in a practical work.

It had been believed that PCNA type was uniquely developed only in Japan. Recently, however, a PCNA-type cultivar, 'Luo Tian Tian Shi', was found growing in Luo Tian prefecture of China (Wang 1982; Wang et al. 1997). Phylogenetic tree based on AFLP analysis showed distant relationship between 'Luo Tian Tian Shi' and Japanese PCNA-type cultivar (Kanzaki et al. 2000a) and it would indicate independent occurrence of each Chinese- and Japanese- PCNA type. Also, the genetic nature of Chinese PCNA trait seemed to be different from that of Japanese PCNA trait. The RFLP marker linked to *Ast* locus could be detected in 'Luo Tian Tian Shi' (Kanzaki et al. 2000b) and Ikegami et al. (2004) reported that hybrids between 'Luo Tian Tian Shi' and Japanese PCNA-type cultivar segregated into PCNA and non-PCNA plants. These results suggest that the gene controlling Chinese PCNA trait should be different from *Ast* gene of Japanese cultivar, although genetic nature of Chinese-PCNA trait has not been understood well. However, as the cross between 'Luo Tian Tian Shi' and Japanese non-PCNA cultivar yield PCNA-type offspring in F_1 generation (Ikegami et al. 2006), Chinese PCNA cultivar will be an important breeding source for persimmon breeding project in the future.

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