Application of Marker-assisted Selection in Persimmon Breeding of PCNA Offspring Using SCAR Markers among the Population from the Cross between Non-PCNA ‘Taigetsu’ and PCNA ‘Kanshu’

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Abstract. Persimmon (*Diospyros kaki* Thunb) is hexaploid, and the pollination-constant, non-astringent (PCNA)/non-PCNA trait of Japanese origin is qualitatively controlled by the *AST/ast* alleles at a single locus and the PCNA trait is recessive to the non-PCNA trait. To avoid inbreeding depression led by repeated crosses among PCNA genotypes, non-PCNA genotypes should be used as cross parents. The marker-assisted selection system has been developed for the selection of PCNA offspring in the progeny derived from the cross of non-PCNA ‘Taigetsu’ (non-PCNA ‘Kurokuma’ × PCNA ‘Taishu’) to PCNA ‘Kanshu’. The primer pairs E8.5/E9r and 7H9F/AST-R were used for detecting the molecular markers A₁ and A₃, respectively, which link *AST* alleles. Complete agreement was found between the sequence-characterized amplified region (SCAR) marker genotype and fruit astringency phenotype of the 48 offspring. The result confirmed that the marker-assisted selection using those markers was highly practical. In a larger offspring population (522 offspring) from the same cross, offspring segregated into 100 with both markers, 162 with only A₁, 179 with A₃, and 81 with neither, and this segregation ratio was significantly different from 2:3:3:2, which is the segregation ratio of random chromosome assortment in autohexaploid. The percentage of offspring expected to be PCNA was 15.5% (81 of 522), which was slightly lower than 20%.

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Persimmon (*Diospyros kaki* Thunb.) cultivars are classified as astringent or non-astringent based on whether the fruits lose their astringency naturally in the mature stage as they mature on the tree. Each type is further classified into two subtypes: variant and constant type depending on the relationship between presence of seeds and flesh color (Hume, 1914; Kajiuira, 1946; Kitagawa and Glucina, 1984; Yonemori et al., 2000). The four types are as follows: 1) PCNA; 2) pollination-variant, non-astringent; 3) pollination-constant, astringent; and 4) pollination-variant, astringent. Among them, the PCNA type is the most desirable for fruit production because this type loses fruit astringency on the tree as part of fruit development, irrespective of seed formation. The PCNA trait of Japanese origin is qualitatively inherited and recessive to the non-PCNA

trait (Ikeda et al., 1985; Yamada, 2005). Almost no PCNA F₁ offspring were obtained from crosses between PCNA and non-PCNA local cultivars or from crosses among non-PCNA local cultivars of Japanese origin (Ikeda et al., 1985). When a non-PCNA cultivar, Nishimurawase, was crossed with six PCNA cultivars and selections, no PCNA offspring were yielded among 95 offspring (Yamada and Sato, 2002).

Therefore, in the breeding program of the National Agriculture and Food Research Organization Institute of Fruit Tree Science (NIFTS), crosses have been made primarily among PCNA cultivars/selections to obtain PCNA offspring. However, repeated crosses among PCNA cultivars/selections has led to inbreeding depression for tree vigor, productivity, and fruit weight (Yamada, 1993, 2005). To avoid this inbreeding depression, the backcross approach with non-PCNA cultivars/selections has also been used for PCNA breeding. ‘Taigetsu’ and ‘Taiten’ are new non-PCNA cultivars selected from a cross between ‘Kurokuma’ (non-PCNA) and ‘Taishu’ (PCNA) (Yamada et al., 2012a, 2012b). The trees of both cultivars are vigorous and highly productive, and their fruits are very large. Accordingly, the use of these cultivars as cross parents could aid in eliminating the inbreeding depression. However, the backcross of non-PCNA F₁ offspring derived from PCNA × non-PCNA to PCNA yielded only 14.5% PCNA offspring on average (Ikeda et al., 1985). So marker-assisted selection should be developed for selecting PCNA offspring efficiently.

Most persimmon cultivars are hexaploid (Tamura et al., 1998), and segregation analysis of molecular markers has indicated hexasomic inheritance with six alleles in the single *AST* locus (Akagi et al., 2010; Kanzaki et al., 2008). The presence of one dominant *AST* allele is sufficient to express the non-PCNA trait, and the PCNA trait is expressed only when all of the alleles present at the locus are recessive *ast* alleles (denoted as aaaaaa). Molecular markers linked to *AST* alleles are reported and these *AST*-linked regions have polymorphism. To date, three types of molecular markers linked to *AST* alleles, A₁, A₂, and A₃, have been reported (Kanzaki et al., 2008, 2009, 2010). The genotype of ‘Kurokuma’ is reported to be A₁A₂A₃aaa, and those of ‘Taigetsu’ and ‘Taiten’ are A₁A₃aaaa and A₂A₃aaaa, respectively (Akagi et al., 2012; Kanzaki et al., 2009, 2010). The astringency types of the offspring populations from the cross between ‘Taiten’ and PCNA have been identified by two SCAR markers (Mitani et al., 2014).

The purpose of the present study is to confirm that SCAR markers could reliably distinguish PCNA and non-PCNA genotypes in a large number of offspring derived from the backcross between ‘Taigetsu’ and PCNA ‘Kanshu’. Furthermore, to reveal the mode of inheritance for the *AST* locus, we estimate the

Fig. 1. Schematic diagram of *Ast*-linked genomic regions. The black boxes indicate the initially isolated random amplified fragment polymorphism (RFLP) fragment, and the numbers in the A_1 and A_2 regions show primer positions from the left end of the isolated amplified fragment length polymorphism (AFLP) fragment (Kanzaki et al., 2001, 2009). E, M, and H represent the *EcoRI*, *MseI*, and *HindIII* restriction enzyme sites, respectively. The light gray boxes indicate the retrotransposon-like inserts. The dotted region in A_3 indicates the large deletion (Akagi et al., 2010, 2012).

Fig. 2. Segregation of the SCAR markers in the persimmon progeny from 'Taigetsu' $\times$ 'Kanshu.' Lanes 1, 3, 4, 7: offspring presumed to be non-PCNA displaying both the A_1 and A_3 markers. Lane 9: offspring presumed to be non-PCNA displaying the A_1 . Lanes 2, 5, 8: offspring presumed to be non-PCNA displaying A_3 . Lanes 6: offspring presumed to be PCNA. M: molecular marker (50 bp ladder; Roche Diagnostics, Tokyo, Japan). SCAR = sequence-characterized amplified region; PCNA = pollination-constant, non-astringent.

segregation ratio of PCNA and non-PCNA offspring in offspring populations.

Materials and Methods

Plant materials. All of the plant materials were persimmon seedlings derived from a cross between 'Taigetsu' (seed parent) and 'Kanshu' (pollen parent). 'Taigetsu' was selected among the F_1 offspring populations from a cross of 'Kurokuma' (non-PCNA) and 'Taishu' (PCNA) and released in 2009 (Yamada et al., 2012a). 'Taigetsu' is a non-PCNA cultivar with large

fruits, increased productivity, and high eating quality. 'Kanshu' is a PCNA cultivar, which resulted from a cross of 'Shinshu' and ['Fuyu' $\times$ ('Okugosho' $\times$ 'Hanagosho')] (Yamada et al., 2006).

The seedlings were grown in a plastic house, and 1-year-old shoots were top-grafted onto mature 'Fuyu' trees in the persimmon breeding selection field at the NIFTS orchard (Higashihiroshima, Hiroshima, Japan).

The fruit astringency/non-astringency types of the fruit-bearing offspring were identified by a sensory (taste) test and the observations of

flesh color. For pollination-variant cultivars, fruits with many seeds are determined to be non-astringent, whereas fruits with only a few seeds were astringent and had many brown specks around the seeds. PCNA flesh is determined to be non-astringent irrespective of the number of seeds with or without brown specks.

PCR for the detection of the SCAR markers. 'Taigetsu' was found to possess two *AST*-linked regions, A_1 and A_3 , and in our previous report, E4/E9r was used to detect the A_1 region, and 7H9F/AST-R was

Table 1. Comparison of the A₁ marker genotype by the E8.5/E9r primers with fruit astringency phenotype of the grafted and fruit-bearing offspring.

A ₁ genotype	Number of grafted offspring	Number of fruit-bearing offspring	Phenotype	
			PCNA	non-PCNA
+	40	22	0	22
–	46	26	11	15

PCNA = pollination-constant, non-astringent.

Table 2. Marker genotypes observed in the offspring population derived from ‘Taigetsu’ and ‘Kanshu’ by polymerase chain reaction with the E8.5/E9r and 7H9F/AST-R primers.

Number of offspring	Marker genotype ^a				The rate of the expected PCNA offspring (%)
	A ₁ +A ₃ +	A ₁ +A ₃ –	A ₁ –A ₃ +	A ₁ –A ₃ –	
522	100	162	179	81	15.5

^aFour genotypes are represented as follows. A₁+A₃+, which exhibit both the A₁ and A₃ markers; A₁+A₃–, which exhibit only the A₁ marker; A₁–A₃+, which exhibit only the A₃ marker; and A₁–A₃–, which exhibit neither marker.

PCNA = pollination-constant, non-astringent.

used to detect the A₃ region (Kanzaki et al., 2009, 2010). In the current study, for detecting the A₁, we developed a separate E8.5/E9r primer pair (E8.5: 5′-CCAATGGAAGAAG GAATTGGAGAGC-3′, E9r: 5′-GCTTAGT CAGCTTAGCCACGCCATTTC-3′) based on the sequence between the E8 and E9 regions (Kanzaki et al., 2009; see Fig. 1, GenBank Accession no. AB428737). For detecting the A₃, the primer pair 7H9F/AST-R, which was designed to amplify the *AST*-linked fragment (Kanzaki et al., 2010; Mitani et al., 2014), was used.

Total DNA was extracted from ≈1 cm² of young leaves, sampled from each seedling or grafted plant from offspring plants, using the Nucleon Phytopure plant DNA extraction kit (GE Healthcare UK Ltd., Buckinghamshire, UK). Polymerase chain reaction (PCR) was performed on a total volume of 10 μL containing 0.2 mM of each dNTP, 0.25 U of Ex Taq (Takara Bio, Shiga, Japan), 0.5 μM of each primer, 1× reaction buffer, and 50 ng of the total DNA sample. The PCR conditions were as follows: initial denaturation at 94 °C for 30 s followed by 40 cycles of 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 20 s for E8.5/E9r or 30 s for 7H9F/AST-R and a final additional extension at 72 °C for 5 min. The amplified PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide.

Results and Discussion

At the beginning of the study, we could detect only the SCAR markers A₁ and A₂ linked to the dominant *AST* alleles in ‘Kur-okuma’, although it has another *AST*-linked region, A₃ (Kanzaki et al., 2009). First, we intended to select non-PCNA offspring derived from the cross between ‘Taigetsu’ and ‘Kanshu’, depending on whether the offspring exhibited the A₁ marker. A 2.1-kb fragment was generated in the offspring possessing the A₁ region using the E4/E9r primer pair (Kanzaki et al., 2009). In this study, the newly developed E8.5/E9r primer pair was used to shorten the reaction time. A fragment of ≈160 bp fragment was also amplified in those offspring with E8.5/E9r

(Fig. 2), indicating that the results using the E8.5/E9r primer pair agreed with the results obtained using the E4/E9r primer pair. Consequently, it was concluded that E8.5/E9r could be used for detecting the A₁ marker.

Forty offspring exhibiting the A₁ marker (A₁+) and 46 offspring that did not display the A₁ marker (A₁–) were grafted onto branches of mature trees. The resulting fruits were subjected to the sensory test to confirm the astringency trait. Four years after grafting, 22 of the A₁+ offspring bore fruits, all of which were found to have the non-PCNA phenotype (Table 1). In contrast, among the resulting 26 fruit-bearing A₁– offspring, 11 and 15 were found to have the PCNA and non-PCNA phenotype, respectively. The segregation ratio (11:15) was not significantly different from 1:1 ($P = 0.433$ by χ^2 test). All of the 15 non-PCNA offspring were later confirmed by the 7H9F/AST-R primers to possess the A₃ marker. The results demonstrated good agreement between the discrimination by SCAR markers and the fruit astringency trait. From these results in addition to the previous study (Mitani et al., 2014), it was found that the detection of the A₁, A₂, and A₃ markers among offspring derived from the backcross of non-PCNA F₁ offspring to PCNA with three primer pairs could be accurate, although the genotype of the non-PCNA cross parents should be elucidated in advance.

A serious problem for breeding new PCNA cultivars is inbreeding depression caused by narrow genetic pools among Japanese PCNA cultivars (Yamada, 1993, 2005). So the backcross of non-PCNA F₁ offspring derived from PCNA × non-PCNA to PCNA should be adopted for PCNA breeding. However, even in the case of the crosses among PCNA cultivars/selections, it takes more than 10 years from the cross to the release of a new cultivar and would take longer in the case of the backcrosses. Thus, the development of markers that can discriminate PCNA from non-PCNA is indispensable.

Subsequently, the applicability of using the E8.5/E9r and 7H9F/AST-R primer pairs for the selection of PCNA offspring in the

breeding program was evaluated. Among 522 offspring, 100 offspring exhibited both the A₁ and A₃ markers (Table 2). Furthermore, 162 offspring displayed only the A₁ marker, and 179 showed only the A₃ marker. Eighty-one offspring did not display either of the markers. The percentage of offspring that were presumed to be the PCNA genotype was 15.5% (81 of 522), which was not significantly different from 18.3% (46 of 251) observed for ‘Taiten’ × ‘Kanshu’ (Mitani et al., 2014) ($P = 0.377$).

The autohexaploid model for inheritance of the *AST* locus was generally fitted to the expected ration with some exceptions using marker genotyping (Akagi et al., 2009). The theoretical offspring segregation ratio from A₁A₃aaaa × aaaaaa is 20% A₁A₃aaaa, 30% A₁aaaa, 30% A₃aaaa, and 20% aaaaaa in random chromosome assortment for autohexaploid, which approached but was significantly different from the observed data in this study ($P = 0.032$). The observed percentage of nulliplex expressing PCNA was 15.5% (Table 2), which was slightly lower than 20%, but the percentage of PCNA offspring was 22.9% (11 of 48) for phenotypic segregation of the astringency type (Table 1), which was not significantly different from 20% ($P = 0.613$ by χ^2 test). Sato et al. (2013) reported that PCNA offspring from the cross between ‘Taigetsu’ or ‘Taiten’ with ‘Kanshu’ had significantly small fruit weight and were likely to cause fruit cracking at the fruit apex, and it is possible that some unknown factors modify the segregation ratio. However, ≈20% PCNA offspring were obtained from the cross of ‘Taigetsu’ and ‘Kanshu’, and it was concluded that those offspring were discriminated effectively by the molecular markers.

In conclusion, a practical application of marker-assisted selection in the PCNA persimmon-breeding program at NIFTS has been shown. PCNA offspring can be selected by the two PCR primers E8.5/E9r and 7H9F/AST-R in the progeny derived from ‘Taigetsu’ × ‘Kanshu.’ With the marker-assisted selection, only scions of PCNA offspring selected from the backcross can be grafted onto mature trees to promote early fruiting in a selection field. Assuming that the backcross of the non-PCNA F₁ offspring derived from PCNA × non-PCNA to PCNA yielded 20% PCNA offspring, the selection efficiency could be increased ≈5-fold and the cost of maintaining fields and trees reduced. It is no doubt that raising a large number of offspring continuously in the field and evaluations using actual trees and fruits are necessary. The marker-assisted selection described in this article will help persimmon breeders to select only PCNA offspring and to improve the efficiency of PCNA persimmon breeding.

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