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RESEARCH

Accurate marker-assisted selection for non-astringent persimmon using a CAPS marker to complement a SCAR marker

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Abstract Persimmon is classified as either pollination-constant non-astringent (PCNA) or non-PCNA on the basis of the loss of astringency in fruit. PCNA trait of persimmon has attracted much research attention owing to its economical merit, as there is no cost in removing astringency. To efficiently develop new PCNA cultivars in crossbreeding, marker-assisted selection plays a crucial role. Here, we describe a cleaved amplified polymorphic sequence (CAPS) marker that can be used to select PCNA persimmon. A sequence-characterized amplified region (SCAR) marker used to screen for PCNA offspring is unsuitable for populations recently derived from two non-PCNA parents ('Yoshidagosho' and 'Toyoichi'), resulting in the selection of non-PCNA offspring as PCNA. To detect specific polymorphisms for these non-PCNA offspring, we analyzed fragment sizes of SCAR marker products by capillary DNA sequencing. A slightly lower-molecular-weight fragment at 353 bp was specifically detected in non-PCNA offspring but not in PCNA offspring. We treated the SCAR marker products with the StuI restriction enzyme and demonstrated that the smaller 353-bp fragment corresponded to allele $a^{353\text{-}1}$, one of four previously identified sequence polymorphisms at the 353-bp peak, and $a^{353\text{-}1}$ is linked to non-PCNA trait. Comprehensive analysis of 130 germplasms by the CAPS marker, detecting the truncated fragment after StuI treatment, indicated the presence of $a^{353\text{-}1}$ in 38 non-PCNA cultivars. Our findings suggest the potential use of the CAPS marker for selecting PCNA offspring derived from these 38 non-PCNA cultivars.

Keywords Astringency · Diospyros kaki · DNA marker · Fruit tree · Marker · MAS

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Introduction

The Oriental persimmon (*Diospyros kaki* Thunb.) has long been grown in China, Korea, and Japan as an important fruit tree, and its popularity is increasing in worldwide. The genus *Diospyros,* contains several horticulturally and economically important

species (*D. kaki* Thunb., *D. oleifera* Cheng, *D. lotus* L., *D. rhombifolia* Hemsl., *D. virginiana* L.), among which *D. kaki* has a significant global market. The total global fruit production of *D. kaki* has increased by more than 400% over 50 years (from 913 976 t in 1970 to 4 866 804 t in 2021) (FAO 2021; MAPA 2021).

It is likely that persimmon was originally astringent, because most East Asian cultivars are astringent, and generally remain inedible without artificial deastringency treatment. Thus, the absence of astringency in mature fruits is an important target in persimmon breeding (Badenes et al. 2013; Sato and Yamada 2016; Ma et al. 2018; Zhu et al. 2019). It's astringency arises from large amounts of proanthocyanidins (condensed tannins) in tannin cells (Taira 1996). Normally, these cells enlarge while accumulating proanthocyanidins until the middle stage of fruit development, whereas those of pollination-constant non-astringent (PCNA)-type fruits cease enlarging at the early stage of fruit development. Dilution of these early proanthocyanidins caused by prolonged fruit enlargement is thought to be a main factor in the natural loss of astringency (Yonemori and Matsushima 1985).

PCNA fruit can be eaten fresh with no need for astringency removal. In addition, they generally have a longer shelf life than deastringency-treated non-PCNA fruit: that of PCNA 'Fuyu' was 28 days, while that of non-PCNA 'Hiratanenashi' treated by the Constant-Temperature Short Duration method was 11 days (Yamada et al. 2012). These commercial merits enhance the need to develop new PCNA persimmon cultivars.

One form of the PCNA trait arose in Japan and is inherited qualitatively and recessively (Ikeda et al. 1985). A single locus, *AST* ("*A*"), controls the trait, and its alleles generally follow hexasomic inheritance model (Akagi et al. 2012). Cultivated persimmon is mostly hexaploid (Tamura et al. 1998), thus expression of the PCNA trait requires a mutant allele of *ast* ("*a*") on each of the six corresponding chromosomes (Akagi et al. 2009, 2010). Another form of PCNA, called Chinese PCNA, which is dominant and is regulated by a different locus from *AST* locus, is found in the Chinese local cultivar 'Luo-Tian-Tian-Shi' (Wang 1982; Ikegami et al. 2006). Unfortunately, this type often retains slight to medium astringency at the Grape and Persimmon Research Station of NARO (Higashihiroshima, Japan), where most Japanese PCNA types completely lose their astringency. Thus, further studies are needed to release new commercially acceptable cultivars (Sato and Yamada 2016). We here describe the Japanese PCNA trait.

Because the recessive inheritance of the PCNA trait substantially reduces the segregation rate of PCNA F_1 offspring in non-PCNA × PCNA crosses (Ikeda et al. 1985), PCNA × PCNA crosses are preferred in developing new PCNA cultivars. However, the recurrent use of such crosses accelerates inbreeding: the number of native PCNA cultivars, except for bud mutants, collected throughout Japan had been limited to only 18 among 600 germplasms at NARO (Yamada 2005). In the late 1980s, NARO's persimmon breeding program faced severe inbreeding depression, seen as weakened tree vigor and small fruit size (Onoue et al. 2022a; Yamada 1993). Breeders thus had to use non-PCNA parents, which have far genetic background from PCNA ones (Naval et al. 2010; Parfitt et al. 2015), to mitigate the inbreeding depression. In the 1990s, NARO focused on pseudo-backcrosses (Sato and Yamada 2016), where non-PCNA × PCNA–derived BC_0 offspring with three or more *ast* alleles are crossed again to a PCNA parent with six *ast* alleles. This 'detour' strategy both mitigates inbreeding and generates a substantial number of PCNA offspring, around 15% in the BC_1 generation (Ikeda et al. 1985).

In addition to the pseudo-backcross strategy, a key to efficient PCNA breeding is to screen offspring at the seedling stage by using molecular markers linked to the *AST* locus. In 'traditional' fruit tree breeding, screening based on fruit traits is not possible until seedlings are physiologically mature. In the case of persimmon, this juvenile phase usually takes 3–5 year even when top-grafting 1-year-old seedling scions onto mature trees (Yamada et al. 2012). Marker-assisted selection (MAS) plays a crucial role in plant breeding programs by facilitating the early identification of desirable seedlings. This results in a reduction in the cost, time, and space necessary for raising genetically unfavorable seedlings, making it especially beneficial for perennial crops (Edge-Garza et al. 2015; Migicovsky and Myles 2017; Ru et al. 2015). In persimmon, a sequence-characterized amplified region (SCAR) marker linked to the *AST* locus was developed by designing primers in the region

flanking a large insertion/deletion called Indel-3 (Kanzaki et al. 2009, 2010). This SCAR marker amplified fragments linked to *AST/ast* (*A/a*) alleles in practical breeding populations, enabling breeders to screen for PCNA individuals (Mitani et al. 2014a, b; Sato and Yamada 2016). Multiplex PCR (Kanzaki et al. 2010), now used in the breeding programs (Sato and Yamada 2016; Blasco et al. 2020) uses two forward and one reverse primers, which amplify both *AST*- and *ast*-linked regions simultaneously (Fig. 1A). These regions have high sequence diversity. Numerous fragment size polymorphisms of *AST*- and *ast*-linked regions were shown by analyzing SCAR marker products on a capillary DNA sequencer (Kono et al. 2016). Comprehensive analysis of 237 germplasms revealed 21 *AST*- and 5 *ast*-linked (a^{347}, a^{349}, a^{351}, a^{353}, and a^{355}) fragments of different sizes. Sequencing the *ast*-linked fragments revealed that the 353-bp fragment has four sequence polymorphisms ($a^{353\text{-}1}$, $a^{353\text{-}2a}$, $a^{353\text{-}2b}$, and $a^{353\text{-}2c}$) (Onoue et al. 2018).

MAS at NARO distinguished over 18000 seedlings by 2020. However, the conventional SCAR marker identifies non-PCNA offspring as PCNA in recent populations derived from the non-PCNA parents 'Yoshidagosho' and 'Toyoichi', thus we need a new method to accurately identify PCNA offspring. These non-PCNA accessions were preferentially used among 130 non-PCNA germplasms as cross-parents because they potentially have fewer *AST* and more *ast* alleles, with estimated *AST*/*ast* dosage genotypes of 2 *AST*, 3 *ast* and 1 unknown allele in 'Yoshidagosho' (*AAaaaX*) and 2 *AST*, 2 *ast* and 2 unknown alleles in 'Toyoichi' (*AAaaXX*), in contrast to means of 3.4 *AST* and 1.5 *ast* in the above populations (Kono et al. 2016; Onoue et al. 2018). Crosses between these cultivars and a PCNA accession would be expected to produce non-PCNA offspring with fewer *AST* and more *ast* alleles, and even PCNA offspring in the BC_0 generation in the pseudo-backcross strategy. The use of yet-unused non-PCNA germplasms in breeding is expanding to mitigate inbreeding, and it is possible that the SCAR markers will sometimes be inapplicable. Here, using F_1 populations derived from 'Yoshidagosho' and 'Toyoichi', we inspected specific polymorphisms in the non-PCNA offspring, developed a new molecular marker detecting the target polymorphism, and report the prevalence of this polymorphism among non-PCNA germplasms.

Materials and methods

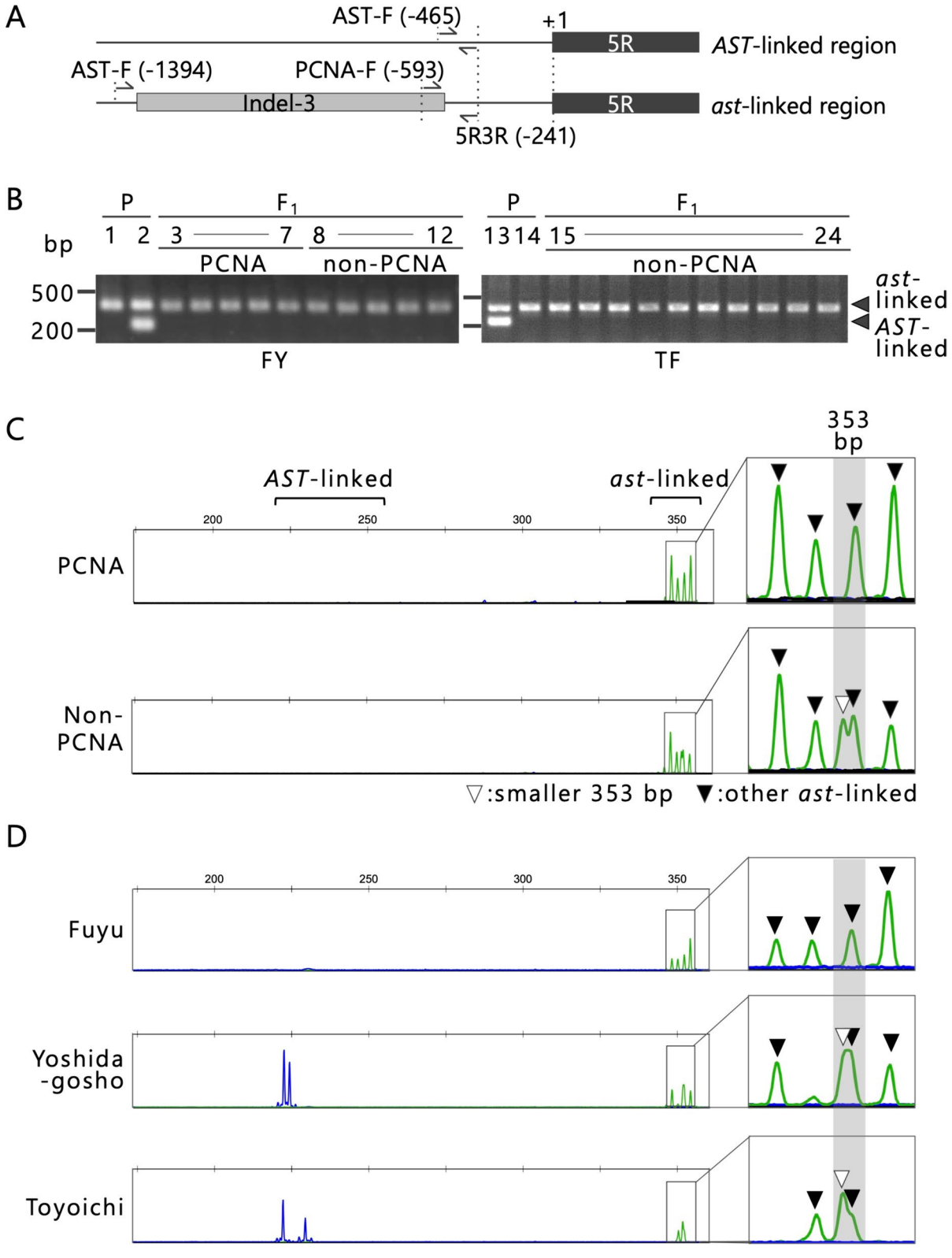
Plant materials

We used two F_1 populations, TF ['Toyoichi' (non-PCNA) × 'Fuyu' (PCNA)] and FY ['Fuyu' (PCNA) × 'Yoshidagosho' (non-PCNA)], which were produced by cross-pollination in 2014 and 2016, respectively. Seeds were extracted from mature fruits of the parent trees and 199 (TF) and 500 (FY) seeds were sown in nursery beds. Germinated 165 (TF) and 365 (FY) seedlings were screened by the multiplex PCR for the SCAR marker (Kanzaki et al. 2010) in combination with a crude-sample PCR method (Onoue et al. 2022b) to select only PCNA offspring of the F_1 seedlings. The SCAR marker indicated 131 non-PCNA, 25 PCNA, and 9 unidentified seedlings for TF; 309 non-PCNA, 55 PCNA, and 1 unidentified seedlings for FY. Finally, scions of 25 (TF) and 52 (FY) 1-year-old seedlings, expected to be PCNA, were grafted onto mature 'Fuyu' trees in a trial field in the next April.

For comprehensive analysis of germplasms, we chose 127 non-PCNA and 3 Chinese PCNA accessions (Table S1) to update the data of our previous study (Onoue et al. 2018). We grew all materials at the Grape and Persimmon Research Station, NARO, Higashihiroshima, Japan. Pests and diseases were managed by conventional methods, and irrigation was applied in summer.

DNA extraction

About 1.0cm^2 of leaf of each top-grafted F_1 scion was frozen in liquid N_2 and homogenized in a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). For DNA purification, the crushed leaf underwent three prewashed cycles, and dispersed in 500 µL prewash buffer, consisting of 0.1 M HEPES-NaOH (pH 8.0), 1% (w/v) soluble polyvinylpyrrolidone, and 10 mM dithiothreitol added immediately before application. Following vortexing, the sample was centrifuged at 20,000 × *g*. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with modified AP1 buffer, containing 2% (w/v) soluble polyvinylpyrrolidone.


A
AST-F (-465)
+1
5R
AST-linked region
AST-F (-1394)
PCNA-F (-593)
Indel-3
5R
ast-linked region
5R3R (-241)
B
P
F1
P
F1
bp
1 2 3 7 8 12 13 14 15 24
PCNA
non-PCNA
non-PCNA
500
200
ast-linked
AST-linked
FY
TF
C
353 bp
AST-linked
ast-linked
200 250 300 350
PCNA
Non-PCNA
▽:smaller 353 bp ▼:other ast-linked
D
200 250 300 350
Fuyu
Yoshida -gosho
Toyoichi

◂**Fig. 1** Specific polymorphisms in non-PCNA F_1 offspring selected by SCAR marker as PCNA. **A** Schematic diagram of the SCAR marker in the *AST*-linked region of 'Nishimurawase' and the *ast*-linked region of 'Jiro' (modified from Onoue et al. 2018). Primer pair AST-F/5R3R amplifies *AST*-linked fragments (220–250 bp), and PCNA-F/5R3R amplifies *ast*-linked fragments (~350 bp). **B** Representative results of agarose gel electrophoresis of (left) FY ('Fuyu' × 'Yoshidagosho') and (right) TF ('Toyoichi' × 'Fuyu'). Lane 1, 'Fuyu' (PCNA); lane 2, 'Yoshidagosho' (non-PCNA); lanes 3–7 (PCNA) and lanes 8–12 (non-PCNA), offspring of FY; lane 13, 'Toyoichi' (non-PCNA); lane 14, 'Fuyu' (PCNA); lanes 15–24 (non-PCNA), offspring of TF. **C** Representative results of fragment size analysis of PCNA and non-PCNA offspring of FY and TF. Only non-PCNA offspring showed a smaller 353-bp fragment (nabla) at 353 bp (gray shading). **D** Fragment size analysis of the three parents of the FY and TF populations

Fragment size analysis

Multiplex PCR for the SCAR marker was conducted using a pair of two forward primers—AST-F (5′-GTTGCATCGCATAGCGGGTTTGAGG-3′) and PCNA-F (5′-CCCCTCAGTGGCAGTGCTGC-3′)—and one reverse primer—5R3R (5′-GAAACACTC ATCCGGAGACTTC-3′) (Fig. 1A, Kanzaki et al. 2010). For analysis in an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA), 5′-end fluorescent-tagged forward primers, FAM-AST-F and HEX-PCNA-F, were used. Each 10μL of the PCR mixture comprised 5 μL of 2 × PCR buffer for KOD FX Neo, 0.2 U KOD FX Neo, 0.4 mM dNTPs, 0.4 μM FAM-AST-F, 0.2 μM HEX-PCNA-F, 0.6 μM 5R3R, and 4–8 ng of purified DNA. The PCR cycling conditions involved an initial temperature of 94°C for 2min, followed by 35–40 cycles at 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 30 s. The final step was 68 °C for 7 min. In the *ast*-linked region (Fig 1A), this predominantly amplifies the region between PCNA-F and 5R3R primer (~352bp), because the extension time is shorter than that required to amplify the region between AST-F and 5R3R primer (~1153bp).

The products were separated either in 1.5% agarose gel or on an ABI 3130xl Genetic Analyzer with a 50-cm capillary array and POP-7 polymer (Thermo Fisher). The sizes of amplified fragments were evaluated against GeneScan 500 ROX Dye Size Standard (Thermo Fisher) using GeneMapper v. 5.0 software (Thermo Fisher).

StuI treatment

Onoue et al. (2018) reported four sequence polymorphisms ($a^{353\text{-}1}$, $a^{353\text{-}2a}$, $a^{353\text{-}2b}$, and $a^{353\text{-}2c}$) within the 353-bp fragments (Fig. 2). To identify polymorphisms corresponding to the smaller 353-bp fragment in the 353-bp region (Fig. 1C: ∇; see "Results and discussion" section), we postulated that $a^{353\text{-}1}$, the least AT-rich sequence of the four (Fig. 2; $a^{353\text{-}1}$ 56.98% < $a^{353\text{-}2c}$ 57.55% < $a^{353\text{-}2a}$ 57.83% < $a^{353\text{-}2b}$ 58.12%), is the smaller fragment. This assumption is based on the observation that DNA fragments with AT-rich regions migrate more slowly than other DNA fragments of the same size in polyacrylamide gel (Stellwagen 1983). Consequently, the $a^{353\text{-}1}$ fragment is expected to migrate faster than the others. To test this hypothesis, we analyzed the presence of an $a^{353\text{-}1}$-specific restriction enzyme (StuI) recognition site (Fig. 2). For StuI treatment, PCR products were incubated at 37 °C for > 2 h in 10 μL of reaction mixture [5 μL of PCR mixture, 1 μL of 10 × CutSmart Buffer, and 5 U StuI (New England BioLabs Inc., Ipswich, MA, USA)].

Evaluation of astringency type

Mature fruits, which have orange skin and are moderately soft, were harvested 3–6 years after top-grafting. Astringency type was determined by sensory test (Kajiura 1946; Yonemori et al. 2000). PCNA-type fruits naturally lose their astringency on the tree irrespective of the number of seeds, while non-PCNA-type fruits always retain strong astringency or lose it only if enough seeds are formed. The astringency type of offspring bearing only non-astringent fruit with many seeds (usually 5–8) was determined by visual assessment of the degree of brown speckle and its localization in flesh: non-PCNA flesh tends to have many large and localized brown speckles. When neither sensory nor visual assessment could determine the astringency type, we excluded these offspring from the analysis. Finally, we used the results of astringency evaluation for 43 (FY) and 18 (TF) seedlings.

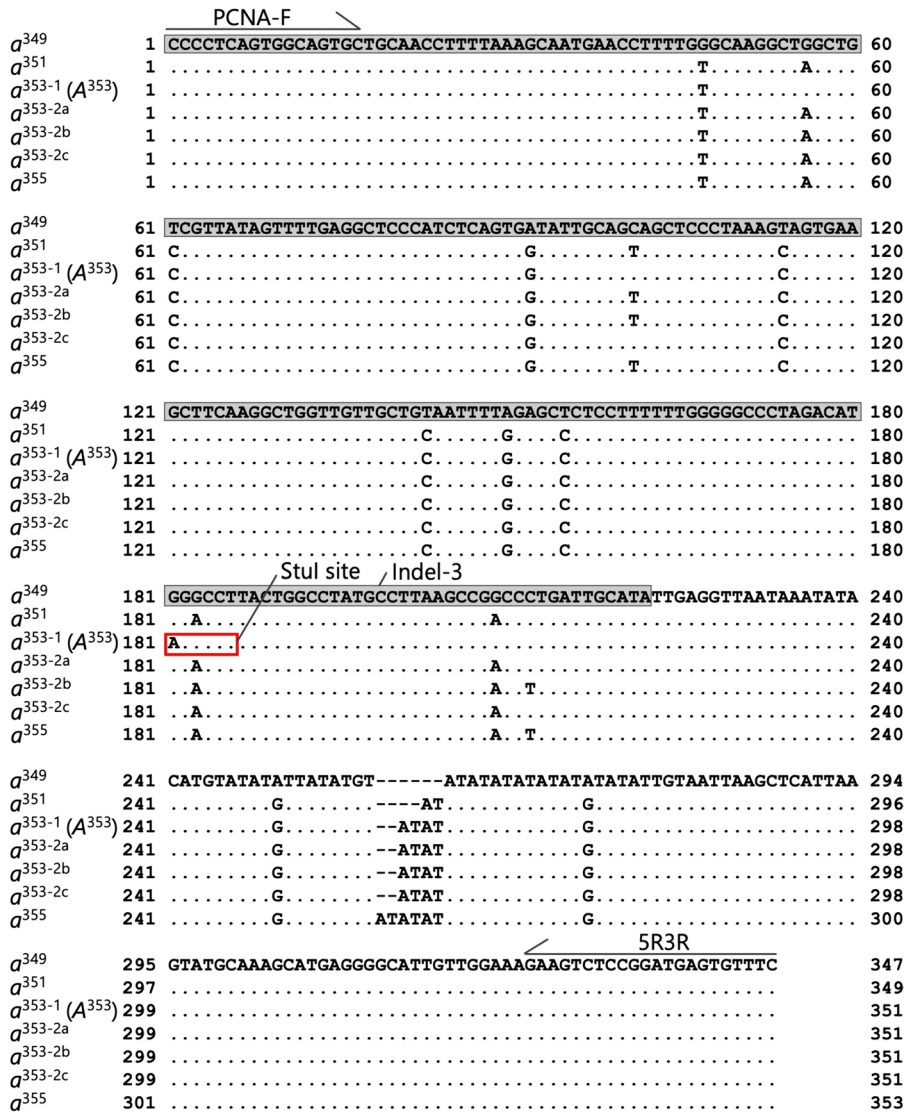


Fig. 2 Nucleotide sequences of the Indel-3-flanking region amplified by the SCAR marker. Seven sequence polymorphisms, consisting of single nucleotide polymorphisms and simple sequence repeats, in the Indel-3-flanking region were amplified by the PCNA-F/5R3R primer set (Fig. 1) (modified from Onoue et al. 2018). Red box indicates $a^{353\text{-}1}$-specific StuI recognition site. Shading indicates a part of the Indel-3 insertion

Results and discussion

Non-PCNA offspring that cannot be distinguished by the conventional SCAR marker

We evaluated the fruit astringency type of the FY and TF F_1 populations at maturity. FY offspring consisted of 8 PCNA and 35 non-PCNA types, and TF offspring consisted of 0 PCNA and 18 non-PCNA types. These results indicate a low accuracy of the SCAR marker in selecting PCNA types, with percentages of 19% (8/43) for FY and 0% (0/18) for TF. In agarose gel electrophoresis of FY and TF, the SCAR marker yielded only an *ast*-linked fragment in the PCNA parent (Fig. 1B: lanes 1 and 14), but both *ast*- and *AST*-linked fragments in the non-PCNA parents (Fig. 1B: lanes 2 and 13). The F_1 offspring of FY and TF showed only a fragment considered to be linked to *ast* (Fig. 1B: lanes 3–7, PCNA; lanes 8–12, non-PCNA; lanes 15–24, non-PCNA). This result confirms that the SCAR maker could not distinguish PCNA from non-PCNA types in these F_1 populations.

To inspect fragment size polymorphisms of these F_1 individuals in detail, we analyzed the SCAR marker products by capillary DNA sequencer (Fig. 1C). All had peaks derived only from the region considered to be linked to *ast* (Fig. 1C). Among them, non-PCNA offsprings specifically

showed a fragment at a slightly lower molecular weight (the smaller 353-bp fragment; Fig. 1C: ∇) in the region corresponding to the 353-bp fragment. To confirm the origin of this smaller fragment, we assessed the fragment sizes of the parents of the two populations (Fig. 1D) and found the smaller 353-bp fragment in both 'Yoshidagosho' (non-PCNA) and 'Toyoichi' (non-PCNA), but not in 'Fuyu' (PCNA), indicating that it was inherited from these non-PCNA parents.

To identify a polymorphism corresponding to the smaller fragment among the four sequence polymorphisms ($a^{353\text{-}1}$, $a^{353\text{-}2a}$, $a^{353\text{-}2b}$, and $a^{353\text{-}2c}$) within the 353-bp fragments (Onoue et al. 2018), we analyzed the presence of an $a^{353\text{-}1}$-specific restriction enzyme (StuI) recognition site (see also Materials and Methods) (Fig. 2). After cleavage by StuI, a new peak appeared at 183 bp in 'Yoshidagosho' and 'Toyoichi', but not in 'Fuyu' (Fig. 3A, grey triangle), and the smaller 353-bp fragment disappeared

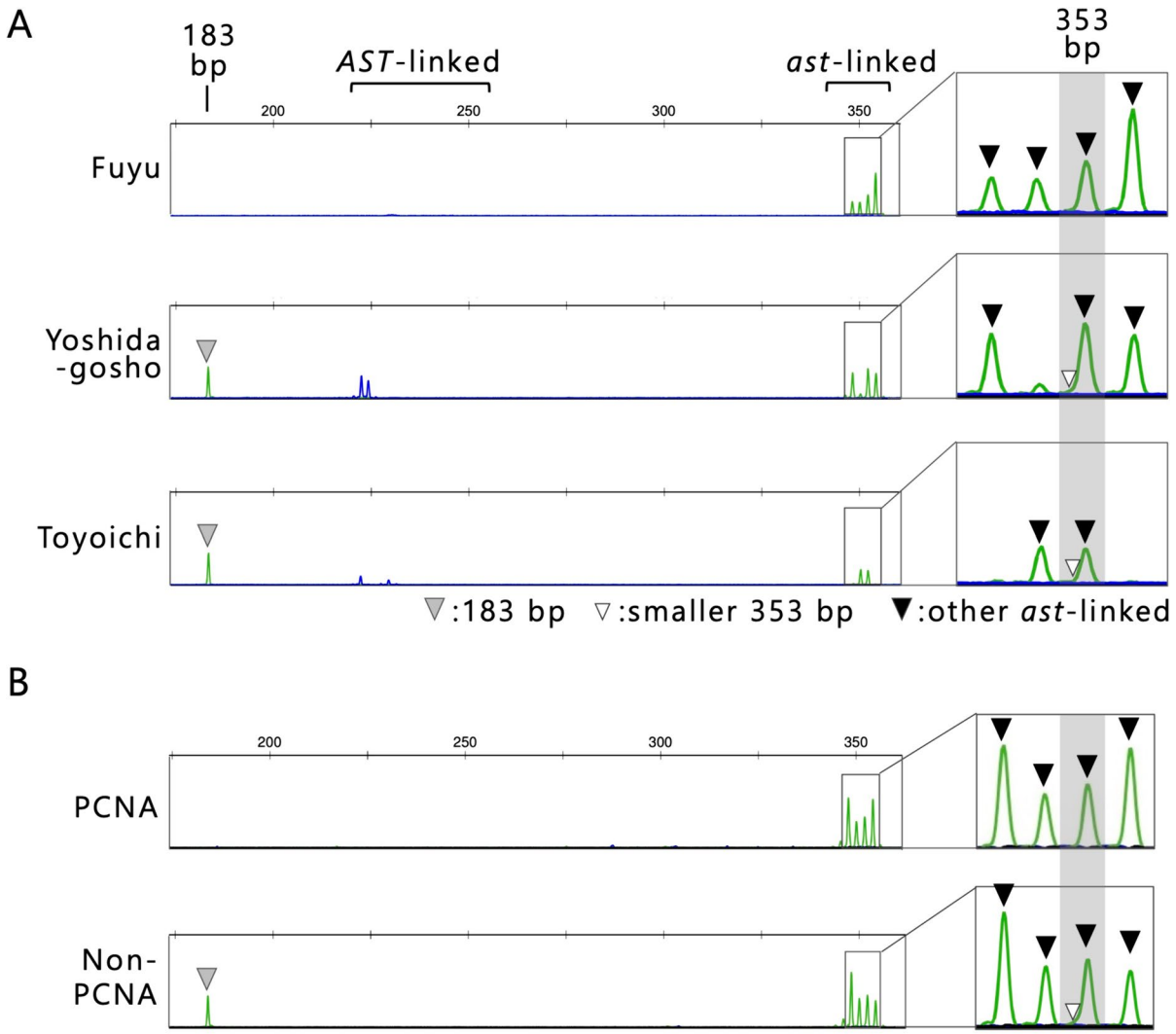


Fig. 3 Examination of the smaller 353-bp fragment by StuI treatment. The same SCAR marker products as in Fig. 1C and D were cleaved with StuI. This treatment specifically yielded a 183-bp peak (grey triangle), used as a CAPS marker, and eliminated the smaller 353-bp fragment (∇) in the 353-bp region (gray shading) in non-PCNA accessions with A^{353}. **A** Fragment analysis of the three parents of the populations FY and TF. **B** Non-PCNA offspring specifically showed the 183-bp peak (grey triangle) (representative results)

(Figs. 1D, 3A:V). In the two F_1 populations, the 183-bp fragment also appeared in non-PCNA offspring but not in PCNA offspring (Fig. 3B, grey triangle). This fragment corresponds to the length of the 5′-DNA fragment when $a^{353\text{-}1}$ is cleaved at the StuI recognition sequence (Fig. 2). The 3′-DNA fragment cannot be detected by capillary DNA sequencer as a loss of the fluorescent label. These results support the hypothesis that $a^{353\text{-}1}$ corresponds to the smaller 353-bp fragment, suggesting that $a^{353\text{-}1}$ (and the smaller fragment) are linked not to *ast* but to *AST*. We refer to $a^{353\text{-}1}$ as A^{353} hereafter. This cleaved amplified polymorphic sequence (CAPS) marker successfully distinguished 35 non-PCNA offspring from 8 PCNA offspring in FY, and 18 non-PCNA offspring in TF populations, with perfect accuracy. This CAPS marker, which assesses the presence of the 183-bp fragment after StuI treatment, will help breeders in accurately screening for PCNA offspring.

The finding of A^{353} linked to the Indel-3 insertion may lead to identifying the *AST* gene. The *ast* allele is thought to be a mutant allele, lacking the ability to promote the accumulation of proanthocyanidins during fruit development owing to a genetic mutation in the *AST* allele (Yonemori et al. 2000; Akagi et al. 2012). Fine genetic mapping has not yet succeeded in isolating the causal gene of *AST* (Nishiyama et al. 2018). Originally, the absence of Indel-3 had been linked to *AST* (non-PCNA) alleles, and its presence to *ast* (PCNA) alleles (Fig. 4A) (Kanzaki et al. 2010; Onoue et al. 2018). This situation raises two possible scenarios for the generation of the *ast* mutation linked to the Indel-3 insertion: the insertion occurred in the *AST*/*ast*-linked region before the *ast* mutation (scenario 1); and the *ast* mutation occurred before the Indel-3 insertion (scenario 2; Fig. 4B). We found the non-PCNA allele of A^{353} linked to Indel-3. So far, no *ast* allele linked to the non-Indel-3 insertion region has been found. These results evidently argue for scenario 1. Although homologous recombination of *AST* and *ast* alleles could have resulted in *AST* (A^{353}) with Indel-3, the fact that no concurrent *ast* without Indel-3 was found suggests the low probability of this scenario. Further analysis is required to determine how the *ast* mutation linked to Indel-3 was generated. Nevertheless, the possible closer genetic relationship of *ast* alleles to A^{353} alleles than to the other *A* alleles implies that comparing genomic regions linked to *ast* alleles with those linked to A^{353} alleles may give clues to identifying the *AST* gene.

Prevalence of A^{353} allele in non-PCNA germplasms

A^{353} ($a^{353\text{-}1}$) was identified in 9 out of 45 non-PCNA accessions with only a single peak linked to the Indel-3 (originally described as linked to *ast*) fragment by dye terminator Sanger sequencing (Onoue et al. 2018). However, presence of A^{353} in the cultivars with multiple peaks of Indel-3-flanking fragments could not be assessed in this way, because dye terminator products derived from multiple fragments yield mixed and complicated sequencing chromatograms (Onoue et al. 2018). Instead, we assessed the presence of A^{353} by CAPS marker in 127 non-PCNA and 3 Chinese PCNA cultivars, some of which have multiple peaks of Indel-3-flanking fragments (Table S1). The 183-bp peak was detected in 38 cultivars, including all 9 cultivars (IDs 6, 11, 15, 20, 28, 31, 32, 34, 36) previously identified to have A^{353} ($a^{353\text{-}1}$) (Tables 1, S1) (Onoue et al. 2018). This finding suggests that crossbred offspring derived from these 38 cultivars, including ‘Yoshidagosho’ and ‘Toyoichi’, inherit A^{353}, which is difficult to identify with the conventional SCAR marker.

These cultivars are potential cross-parents for use in mitigating inbreeding in the breeding population. In NARO’s breeding program, by 2023, 143 offspring derived from the 4 cultivars among the 38 with A^{353} were selected as PCNA offspring by the SCAR marker, but evaluation of astringency type revealed that they were non-PCNA. In such cases, the new CAPS marker could accurately discriminate PCNA from non-PCNA offspring.

Among the 38 cultivars, the genotype of ‘Amahyakume’, which was previously estimated to be *AAAaaa* from multiple polymorphisms generated by the SCAR marker and allele frequency in an F_1 population [‘Amahyakume’ (non-PCNA) × V-13 (PCNA)] (Kono et al. 2016), has been updated to *AAAAaa* (Table S1). The *AAAaaa* genotype implies 5% segregation of PCNA offspring in this F_1 population under hexasomic inheritnce, but no PCNA offspring had emerged (data not shown). The presence of A^{353} in ‘Amahyakume’ corresponds to this fact. The same situation applies to ‘Kurokuma’, whose genotype was previously estimated to be either *AAAaaa* or *AAAaaX* (Akagi

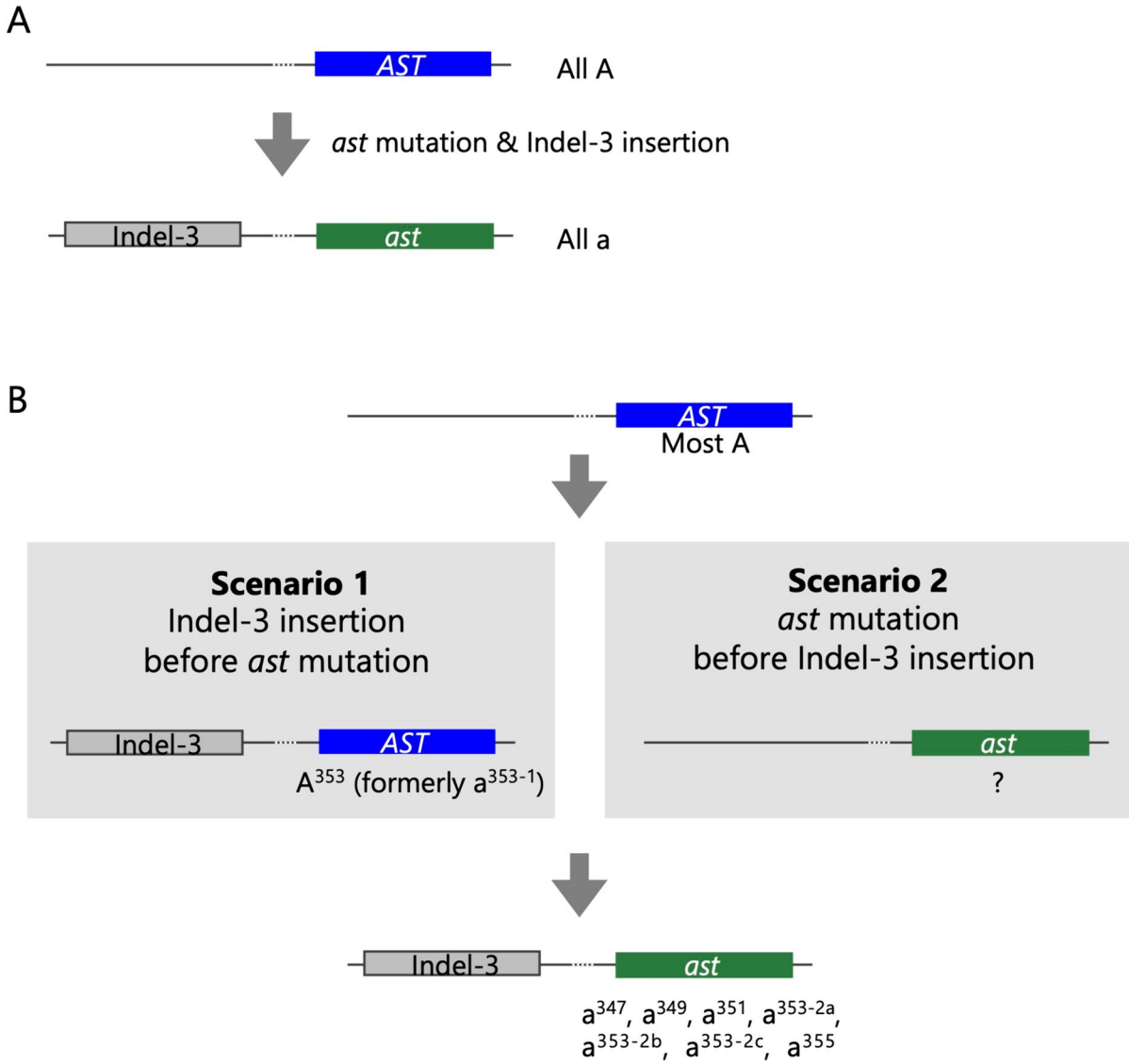


Fig. 4 Possible scenarios for generation of *ast* mutation. **A** Original situation of complete links between absence of Indel-3 and *AST* alleles, and between presence of Indel-3 and *ast* alleles. **B** Scenario 1: Indel-3 insertion occurred in the *AST/ast*-linked region before *ast* mutation. Scenario 2: *ast* mutation occurred before Indel-3 insertion

et al. 2010; Onoue et al. 2018), and has now been updated to *AAAAaa*. Kanzaki et al. (2010) reported no PCNA F_1 offspring in F_1 of 'Kurokuma' (non-PCNA) × 'Taishu' (PCNA). The presence of A^{353} as an *AST*-linked allele in these two parents is in good agreement with the absence of PCNA F_1 offspring in crosses with PCNA parents.

Table 1 List of 38 non-PCNA cultivars with A^{353} allele

ID[a]	Name	ID	Name	ID	Name
1	Aburaden	14	Hoshoumaru	27	Shibumyotan
2	Amahyakume	15	Huo-shi	28	Shinhachiya
3	Beniemon	16	Ichidagaki	29	Shogatsu-Italy
4	Beniwase	17	Ichiryo	30	Toyoichi
5	Ebo	18	Inasa	31	Triumph
6	Eboshi	19	Kanzo (Kanzou)	32	Tsukiyo
7	Egosho	20	Koshumaru	33	Umurbey
8	Gailey	21	Kunitomi	34	Yatsudera
9	Gionbo	22	Kurokuma	35	Yoshidagosho
10	Goshogaki-Sagae	23	Miyazakitanenashi	36	Yotan
11	Harbiye	24	Mizushima	37	Yotsumizo
12	Heixinshi (Hei-xin-shi)	25	Okayamaokugosho	38	Zenjimaru
13	Hiratanenashi	26	Rojo Brillante		

Detected *AST* and *ast* alleles are shown in Table S1

[a]ID is consistent with Table S1

Conclusions and prospects

We successfully developed a new CAPS marker for more reliable MAS in persimmon breeding. Without StuI digestion, it may be possible to distinguish the astringency type by investigating the smaller 353-bp fragment, but the overlapping peaks of the multiple 353-bp fragments, as in 'Yoshidagosho' (Fig. 1D), would necessitate a keen eye to detect it. Finding the 183-bp fragment after StuI treatment for A^{353} will allow more accurate identification of PCNA offspring. Fragment analysis using capillary sequencers in combination with the CAPS marker is, at present, the most practical method to detect these specific fragments. Developing the method for detecting the specific polymorphisms by agarose gel electrophoresis is a future task. This method will be cost-effective and is easier to introduce into the current breeding program than capillary-DNA-sequencer-based fragment analysis. The CAPS marker will be useful in cases where the existing SCAR marker alone cannot distinguish PCNA offspring, as in crosses using the 38 germplasms identified here as having A^{353}. Whether the A^{353} allele derived from these accessions is linked to the non-PCNA trait needs to be investigated. Nevertheless, combining the CAPS marker with the conventional SCAR marker offers the most promising method of identifying PCNA offspring.

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Declarations

Competing interests The authors declare no competing interests.

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