Development of Molecular Markers Linked to the Allele Associated with the Non-astringent Trait of the Chinese Persimmon (*Diospyros kaki* Thunb.)

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Persimmon cultivars are classified into four types depending on the nature of astringency loss of the fruit, namely pollination constant non-astringent (PCNA), pollination variant non-astringent (PVNA), pollination variant astringent (PVA), and pollination constant astringent (PCA). Among these four types, PCNA is the most important cultivar for persimmon breeding due to the stable loss of natural astringency on the tree. The trait of natural astringency loss is recessive in Japanese PCNA cultivars, while that in the Chinese PCNA cultivar, ‘Luo tian tian shi’, is dominant and the latter locus, termed *CPCNA*, is different from that in Japanese cultivars. In order to develop a molecular marker for the selection of the *CPCNA*-type cultivar, we performed amplified fragment length polymorphism (AFLP) in combination with bulked segregant analysis for F₁ offspring derived from ‘Luo tian tian shi’, which was used as the maternal parent. A total of 384 primer combinations were tested, and three AFLP markers, namely EACT-MCCC-222 (RO1), EGGC-MCTC-309 (RO2), and EGCC-MCGA-105 (RO3), linked to the *CPCNA* dominant allele were obtained. Among these markers, EGGC/MCTC-309 (RO2) was converted into a sequence-characterized amplified region (SCAR) marker. PCR analysis using F₁ offspring (n=264) revealed that the relevance ratio of the SCAR marker was 94%. The polymorphism of the RO2 marker, which was strongly linked to the *CPCNA* dominant allele, was detected in only two Chinese PCNA cultivars, namely ‘Luo tian tian shi’ and ‘Tian bao gai’, among the Chinese, Korean, and Japanese cultivars tested. These results indicate that the RO2 marker contributes to marker-assisted selection for breeding programs of new PCNA cultivars having the *CPCNA* trait.

Key Words: amplified fragment length polymorphism, astringency, bulked segregant analysis, *Diospyros kaki*, marker-assisted selection.

Introduction

The cultivated persimmon ($2n = 6x = 90$) fruit accumulates a large amount of condensed tannins/proanthocyanidins (PAs), which cause astringency by coagulating proteins in taste buds and creating a

puckering or drying sensation. The fruits are classified as astringent (A) or non-astringent (NA) based on the loss of natural astringency on the tree. Astringent and non-astringent cultivars are further classified into pollination variant (PV) or pollination constant based on the astringency loss and the presence of seeds (Hume, 1914). Thus, the fruits are classified into the following four types: pollination constant astringent (PCA), pollination constant non-astringent (PCNA), pollination variant astringent (PVA), and pollination variant non-astringent (PVNA) (Yonemori et al., 2000). In PV-type fruit, astringency loss is associated with volatiles, such as ethanol and acetaldehyde generated from seeds (Sugiura and Tomana, 1983). These volatiles promote

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insolubilization (polymerization) of PAs, which causes astringency loss in the persimmon fruit (Tanaka et al., 1994). Among these four types, PCNA fruit is the most desirable for fresh consumption because of the stable loss of astringency on the tree during fruit development (Yonemori et al., 2000). Astringent fruits accumulate PAs in the vacuoles of specific cells termed “tannin cells” and remain astringent until full maturation. In contrast, PCNA fruits terminate PA accumulation in these cells at an early stage of fruit development, thereby causing reduced PA levels and smaller tannin cells than those of non-PCNA (PCA, PVA, and PVNA) fruits (Yonemori and Matsushima, 1985, 1987).

PCNA-type persimmons were thought to have been developed uniquely in Japan; however, a PCNA cultivar, ‘Luo tian tian shi’ was discovered in China (Wang et al., 1982). The Japanese PCNA trait conferred by the *AST* locus is recessive in non-PCNA cultivars (Ikeda et al., 1985; Yamada and Sato, 2002). Since persimmon is hexaploid, crossing between Japanese PCNA and non-PCNA cultivars yielded only non-PCNA offspring, and backcrossing of this F_1 individual with PCNA cultivars yielded only around 15% of PCNA offspring (Ikeda et al., 1985). In addition, the restricted gene pool for PCNA parents causes inbreeding depression. In contrast, crossing tests of Japanese PCNA or non-PCNA cultivars with Chinese PCNA cultivars yielded both astringent and non-astringent F_1 offspring in a nearly 1:1 ratio (Ikegami et al., 2004, 2006). This result suggests that the Chinese PCNA trait controlled by a locus, termed *CPCNA*, is dominant in non-PCNA cultivars; therefore, introducing *CPCNA* in PCNA cultivars in PCNA breeding programs is meaningful not only to expand the genetic background of PCNA breeding, but also to improve the occurrence ratio of PCNA in F_1 offspring.

Marker-assisted selection (MAS) is considered a useful tool in breeding programs, especially for tree crops. Kanzaki et al. (2001) obtained restriction fragment length polymorphism (RFLP) markers linked to the *AST* locus by performing amplified fragment length polymorphism (AFLP) in combination with bulked segregant analysis (BSA), and these markers were later converted into PCR markers (Kanzaki et al., 2009). To develop an MAS system of *CPCNA* for breeding Chinese-type PCNA persimmons, we performed AFLP analysis (Vos et al., 1995) in combination with BSA (Michelmore et al., 1991).

Materials and Methods

Plant materials

Leaves of the F_1 population derived from a cross between Chinese PCNA ‘Luo tian tian shi’ and Japanese PCNA ‘Okugoshō’ (RO family) were used for AFLP analysis. The segregation ratio of PCNA/non-PCNA in RO family is 48:56 ($n=102$). As ‘Luo tian tian shi’ does not have the *ast* allele (Akagi et al., 2010; Kanzaki et al., 2000a), F_1 plants derived from crosses between

‘Luo tian tian shi’ and Japanese PCNA cultivars are heterozygous on the locus (Ikegami et al., 2006); therefore, the PCNA phenotype of offspring segregated in the F_1 population of the cross is not controlled by the *ast* allele, but the *CPCNA* allele. Leaf samples were obtained from seedlings top-grafted and grown in the field of the Grape and Persimmon Research Station, National Institute of Fruit Tree Science, Higashihiroshima, Japan, or from seedlings planted directly into the ground at the Experimental Farm, Kyoto University, Takatsuki, Japan. BSA was performed by pooling DNA of 10 PCNA plants as a PCNA bulk and 10 non-PCNA plants as a non-PCNA bulk.

For evaluation of the SCAR marker, 264 F_1 offspring of ‘Luo tian tian shi’ and other different cultivars were analyzed. F_1 offspring of ‘Luo tian tian shi’ were as follows: 81 F_1 offspring of RO family; 98 F_1 offspring of ‘Luo tian tian shi’ × PCA ‘Yotsumizo’ (RY family); 49 F_1 offspring of ‘Luo tian tian shi’ × PCA ‘Iwasedo’ (RI family); and 36 F_1 offspring of ‘Luo tian tian shi’ × Japanese PCNA ‘Taishu’ (310 family), and the cultivars were as follows: two Chinese PCNA cultivars, ‘Luo tian tian shi’ and ‘Tian bao gai’; five Japanese PCNA cultivars, ‘Fuyu’, ‘Goshō’, ‘Jiro’, ‘Hanagoshō’, and ‘Taishu’; five Japanese non-PCNA cultivars, ‘Amahyakume’, ‘Atago’, ‘Iwasedo’, ‘Saijō’, and ‘Yotsumizo’; and nine Chinese and Korean non-PCNA cultivars, ‘Ban si’ (Korea), ‘Sa kok si’ (Korea), ‘Jin shi’ (China), ‘Huo jing’ (China), ‘Huo shi’ (China), ‘Qu jing shui shi’ (China), ‘Da mo pan’ (China), ‘Hei xin shi’ (China), and ‘Mei xian niu xin shi’ (China). ‘Tian bao gai’ is reported to be a PCNA cultivar found in Luo tian County, Hubei province, China, according to sensory testing and analysis of tannin cell size (Yonemori et al., 2005). All cultivars used were conserved and grown in Akitsu, Grape and Persimmon Research Station, National Institute of Fruit Tree Science.

AFLP analysis

Total genomic DNA was extracted from the leaves of each plant by the CTAB method according to Doyle and Doyle (1987). After purifying DNA by phenol extraction and polyethylene glycol precipitation, DNA concentration was adjusted to $50 \text{ ng} \cdot \mu\text{L}^{-1}$ for AFLP analysis. DNA digestion, adapter ligation, and preamplification were performed as described by Vos et al. (1995) and Kanzaki et al. (2001) using the AFLP analysis system I kit (Invitrogen, USA). Genomic DNA ($1.25 \mu\text{g}$) of each bulked sample, parent, and individual was digested with *EcoRI* and *MseI* in $12.5 \mu\text{L}$ reaction mixture, and *EcoRI* and *MseI* adapters for each restriction site were ligated using T4 DNA ligase, diluted 10 times with TE buffer, and then used as template DNA. After preamplification with $2.5 \mu\text{L}$ template DNA in a total of $25 \mu\text{L}$ reaction volume, selective amplification was performed with primer pairs including three additional selective nucleotides at the 3' end of each primer. A total of 384

combinations of *EcoRI* and *MseI* primers were used for screening PCNA and non-PCNA bulks (Table 1). PCR products of selective amplifications were denatured by boiling with an equal volume of the loading dye (98% formamide, 10 mM ethylenediaminetetraacetic acid, 0.05% bromophenol blue, and 0.05% xylene cyanol), and aliquots were loaded onto 6% polyacrylamide gel. After electrophoresis, fragments were transferred onto the Biodine B membrane (Pall, USA). The membrane was then hybridized with 5' digoxigenin (DIG)-labeled oligonucleotide (Sigma Genosis, Japan), which hybridizes with the *EcoRI* adaptor sequence at 42°C for 2 h. After hybridization, membranes were first washed twice in $2 \times$ SSC containing 0.1% SDS for 5 min at room temperature, and then twice in $0.1 \times$ SSC containing 0.1% SDS for 15 min at 42°C. Target bands were detected using anti-DIG-alkaline phosphatase (Roche Applied Science, USA) and chemiluminescent substrate CDP-star (Roche Applied Science) according to the manufacturer's instructions.

Cloning of AFLP markers

Three AFLP markers (RO1, RO2, and RO3) were detected as candidates, and were recovered from gels electrophoresed and stained using the Silver Sequence™ DNA sequencing system (Promega, USA). Staining was performed according to the manufacturer's instructions. TE buffer (150 µL) was added to each excised marker band, and the bands were frozen and thawed at -20°C and 4°C, respectively. After denaturing by incubation for 15 min at 95°C, samples were centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was then transferred to a fresh tube and precipitated with ethanol, dried in a vacuum, and dissolved in 10 µL TE buffer. After 10 ng of the recovered DNA was reamplified with the same selective primer combination, the reamplified product was subcloned into the pGEM-T easy vector (Promega). DNA sequences of the resultant plasmids were determined with T7 or M13RV primers using the DYEnamic ET terminator cycle sequencing kit (GE Healthcare, USA) and ABI PRISM 310 DNA genetic analyzer (Applied Biosystems, USA).

RFLP analysis

Total genomic DNA (20 µg) from 'Luo tian tian shi', 'Okugoshō', and three offspring of PCNA or non-PCNA was digested with restriction enzymes *Hind* III or *Xba*I, run onto 0.8% agarose gel, transferred onto a Biodyne Plus membrane (Pall), and hybridized with a DIG-labeled probe prepared using the PCR DIG probe synthesis kit (Roche Applied Science). The probe was prepared using the AFLP fragment cloned into the pGEM-T easy vector as a template and primer pairs (forward: 5'-GAGTCCTGAGTAACCCCGCATT-3', reverse: 5'-CGAGTAGTGTGGAGTCAGAGT-3' for RO1; forward: 5'-TCTTTACAATCGCGCCGGGGG-3', reverse: 5'-ACTCATGTTTCGTAACAATGGCC-3' for RO2; forward: 5'-GTACCAATTCGCTGACTGAAA-3', reverse: 5'-TCCTGAGTAACGACGTCACACT-3' for RO3). After hybridization of membranes at 64°C (RO1), 63.5°C (RO2), and 55°C (RO3) for 16 h, the blot was first washed twice in $2 \times$ SSC containing 0.1% SDS for 5 min at 64°C (RO1), 63.5°C (RO2), and 55°C (RO3), and then twice in $0.1 \times$ SSC containing 0.1% SDS for 15 min at 64°C (RO1), 63.5°C (RO2), and 55°C (RO3). Target signals were detected as described for AFLP analysis.

Evaluation of the SCAR marker

A SCAR marker was designed from the RO2 sequence. The PCR reaction mixture consisted of 10 ng template DNA, $1 \times$ reaction buffer (supplied with Takara *rTaq* polymerase; Takara Bio, Japan), 200 µM dNTPs, 0.2 µM of a designed primer pair (2-2F: 5'-TCTT TACAAGTGAAGGGGGG-3', 2-1R: 5'-TGTCTTA CAGACGTCGTCGGT-3'), and 1 U *rTaq* DNA polymerase (Takara Bio) in 15 µL reaction volume. A designed primer (2-1F: 5'-CGAAGTAATGTGCCGTA AGCT-3') in place of 2-2F was used as a positive control reaction for non-PCNA-type (see Fig. 3). The PCR reaction with a primer pair (2-1F and 2-2R) gives a 122-bp band both in PCNA and non-PCNA types. The mixture was initially denatured at 95°C for 1 min, followed by 35 cycles of PCR amplification with denaturation for 20 s at 94°C, primer annealing for 20 s at 58.5°C, and primer extension for 30 s at 72°C. Amplified products were separated by electrophoresis on 2% agarose gel with $1 \times$ TAE buffer and stained with ethidium bromide. This SCAR marker was tested for 264 F₁ offspring and cultivars (see *Plant materials*). Based on the presence or absence of the 158-bp band linked to the Chinese PCNA (*CPCNA*) trait, the marker genotype of each F₁ offspring was determined. The phenotype of the fruit was determined based on two parameters deeply related to proanthocyanidin accumulation, tannin cell size, and soluble phenol content, as described in previous reports (Ikegami et al., 2006). The non-PCNA type has larger tannin cells and higher soluble phenol content compared to the PCNA type. The normal distribution of PCNA/non-PCNA in tannin cell size and

Table 1. *EcoRI* and *MseI* primers used for bulked segregant analysis.

<i>EcoRI</i> primers			<i>MseI</i> primers	
EAAG	EGAA	EGGA	MCAA	MCGA
EAAC	EGAT	EGGT	MCAT	MCGT
EAGG	EGAG	EGGG	MCAG	MCGG
EAGC	EGAC	EGGC	MCAC	MCGC
EACA	EGTA	EGCA	MCTA	MCCA
EACT	EGTT	EGCT	MCTT	MCCT
EACG	EGTG	EGCG	MCTG	MCCG
EACC	EGTC	EGCC	MCTC	MCCC

soluble phenol content slightly overlaps; therefore, the assessment of the phenotype may not be precise. However, the trait appears to be independent and no individual showed intermediate tannin cell size and soluble phenol content among the F_1 offspring tested in this analysis.

Results

A total of 384 primer combinations of 24 *Eco*RI and 16 *Mse*I primers were used in BSA against subsets of PCNA and non-PCNA bulks. Ninety-four polymorphic markers present in PCNA and absent in non-PCNA were identified between the two bulks. After these markers were tested using the same individual plants used for bulks and parents, three primer sets (EACT-MCCC, EGCG-MCTC, and EGCG-MCGA) used for AFLP markers and designated RO1 (222 bp), RO2 (309 bp), and RO3 (105 bp), respectively, were present in PCNA but not in non-PCNA offspring (Fig. 1). Sequences of these three fragments (RO1, RO2, and RO3) were determined and used as probes in the following RFLP analysis. RFLP analyses in ‘Okugosho’, ‘Luo tian tian shi’, and six F_1 offspring showed multiple bands in RO1 and RO3; however, only a single band specific to PCNA was detected in RO2 (Fig. 2).

In all RFLP analyses, PCNA-specific polymorphisms were detected (Fig. 2, arrows indicate a PCNA-specific signal). This result suggests that the three AFLP markers are applicable as RFLP markers for distinguishing the PCNA type for the progeny. In order to apply these three markers to a large population, we tried to develop PCR-based markers. Among these markers, the RO2 marker was easily converted to the SCAR marker. As for RO1

Fig. 1. AFLP fingerprints of PCNA and non-PCNA bulks, parents, and individuals for performing bulked segregant analysis (BSA) using primer combination EACT/MCCC-222 (RO1), EGCG-MCTC-309 (RO2), and EGCG-MCGA-105 (RO3). Lanes: L, ‘Luo tian tian shi’; O, ‘Okugosho’; P, PCNA bulk; N, non-PCNA bulk; PCNA, PCNA individuals; non-PCNA, non-PCNA individuals. Arrows indicate AFLP markers and their sizes.

and RO3, SCAR markers were also developed using the flanking sequences in the fosmid clones screened in a ‘Luo tian tian shi’ genomic library; however, the relevance rate of these markers was lower than that of RO2 (T. Akagi, unpublished data). Sequences of the AFLP fragment and a primer set of the SCAR marker for RO2 are given in Figure 3. The resultant SCAR marker for RO2 was a dominant marker linked to the *CPCNA* allele, and amplified the PCNA-specific 158-bp fragment (Fig. 4A), while the 122-bp band with 2-1F and 2-1R was detected both in PCNA and non-PCNA types as a positive control (data not shown). Interestingly, this SCAR marker fragment was detected not only in ‘Luo tian tian shi’ but also in ‘Tian bao gai’ (Fig. 4B). Furthermore, the fact that the 158-bp fragment of the SCAR marker for RO2 was not detected in all Japanese PCNA cultivars tested (Fig. 4B) suggests that the non-astringent trait of all Japanese PCNA cultivars is due to homozygous recessive alleles on the *AST* locus, which is different from alleles on the *CPCNA* locus.

PCR analysis using F_1 offspring ($n = 264$) revealed that the average relevance rate of the SCAR marker was 94% (Table 2). In the three F_1 families, ‘Luo tian tian shi’ × ‘Okugosho’, ‘Luo tian tian shi’ × ‘Yotsumizo’, and

Fig. 2. RFLP analysis of genomic DNA digested with *Xba*I for RO1 and RO2, and *Hind*III for RO3. Lanes: L, ‘Luo tian tian shi’; O, ‘Okugosho’; PCNA, PCNA offspring; non-PCNA, non-PCNA offspring. Arrows indicate RFLP markers and their sizes.

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GGCCACATCAAAAATAGACTTTATCTTCTTCTAACCCCTAAACTCCGATATCCTTCTCTCTAAT
ATCTTTACAAGTCTGAGAGGGGGGCGAGGTGGAAGTCGCGAAGTAATGTCCCGGTAACTCGG
2-2F                                     2-1F
AGTCATCCTTGTATTATTTTGTCTGAGAGACAGCTTGAAGCCTCTACACCTCAAAAATGGTAA
GCTCGGGTTTCGTACCGACGACGCTCTGTAAAGACAAAAGAAAGGCTCGCATAAAAGAAATAA
2-1R
AATTGGGGCCATTGTTACGACCATGAGTTAA

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Fig. 3. Sequence of the AFLP fragment for RO2 and the primer set designed for SCAR.

‘Luo tian tian shi’ × ‘Iwasedo’, the relevance rate were 96.3%, 94.9%, and 95.9%; thus, the occurrence rates of the recombination type were 3.7%, 5.1%, and 4.1%, respectively. The relevance rate in ‘Luo tian tian shi’ × ‘Taishu’ was 83.3%, which was lower than that of the other F_1 families. This result was presumably due to the small population of the latter family ($n=36$). Our results clearly show that the SCAR marker for RO2 is strongly linked to the *CPCNA* allele and that it will greatly contribute to MAS.

Discussion

To our knowledge, this is the first study on genomic analysis of the *CPCNA* allele. Previous reports on the inheritance of the PCNA phenotype in ‘Luo tian tian shi’ (Ikegami et al., 2004, 2006) suggest that the Chinese PCNA phenotype is controlled by a locus different from the PCNA phenotype of Japanese cultivars. In addition, ‘Luo tian tian shi’ is suggested to be a phylogenetically distant relative of Japanese PCNA cultivars, as demonstrated by AFLP analysis (Kanzaki et al., 2000b). The SCAR marker designed from the RO2 sequence

was detected only in ‘Luo tian tian shi’ and ‘Tian bao gai’ among the cultivars tested in this study. The genetic background of ‘Tian bao gai’ with respect to the non-astringent trait has not been explained to date. Our results imply that this trait in ‘Tian bao gai’ is due to the same dominant allele on the *CPCNA* locus as in ‘Luo tian tian shi’. The study also showed a high specificity of RO2 polymorphism linked to the dominant allele on the *CPCNA* locus among cultivars. Analysis using F_1 offspring ($n=264$) also revealed that the recombination value between the SCAR marker and *CPCNA* allele was around 6%; therefore, the SCAR marker for RO2 would enable MAS for the PCNA phenotype derived from the *CPCNA* dominant allele.

Determining the molecular function of the *CPCNA* locus or *CPCNA* dominant allele would be interesting. In Chinese PCNA-type fruits, most of the expression of genes involved in PA biosynthesis, such as anthocyanidin reductase and dihydroflavonol 4-reductase, does not markedly decrease as in Japanese PCNA-type fruits (Ikegami et al., 2005), but genes whose functions in PA biosynthesis remain unknown, such as the genes encoding serine carboxypeptidase-like proteins, 3-dehydroquinase hydratase/shikimate 5-dehydrogenase, glutathione *S*-transferase, and flavonoid 3-galactosyltransferase, decrease coincidentally with the PA level (Ikegami et al., 2009). Identification of this *CPCNA* locus should reveal new aspects of the regulation of biosynthesis of plant flavonoids or secondary metabolites. The dominant character of the *CPCNA* trait suggests that an inhibitor-like function in PA accumulation would exist on this locus (or dominant allele). In this study, we identified three AFLP markers linked to the *CPCNA* allele, which would help identify the *CPCNA* allele by map-based positional cloning. Ikegami et al. (2006) reported that the segregation ratio of PCNA and non-PCNA was nearly 1 : 1 in the F_1 offspring used in this study, and that ‘Luo tian tian shi’ seemed to be simplex at the *CPCNA* locus, suggesting the following three allelic statuses for the *CPCNA* locus: Aaaaaa (autohexaploid), Aaaa (autoallohexaploid), and Aa (autoallohexaploid or allohexaploid). Identifying a locus showing the polyploidy inheritance mode on a genetic map is difficult; however, genome-wide mapping has recently been performed using AFLP markers in the hexaploid sweet potato (*Ipomoea batatas*)

Fig. 4. PCR analysis of markers in the F_1 population (A) and cultivars including Chinese PCNA cultivar ‘Tian bao gai’ (B). A. Lanes: M, 100-bp ladder marker; L, ‘Luo tian tian shi’; O, ‘Okugosho’; PCNA, PCNA individuals; non-PCNA, non-PCNA individuals. B. Lanes: M, 100-bp ladder marker; 1, ‘Luo tian tian shi’; 2, ‘Tian bao gai’; 3, ‘Fuyu’; 4, ‘Gosho’; 5, ‘Jiro’; 6, ‘Hanagosho’; 7, ‘Taishu’; 8, ‘Amahyakume’; 9, ‘Atago’; 10, ‘Iwasedo’; 11, ‘Saijo’; 12, ‘Yotsumizo’; 13, ‘Ban si’; 14, ‘Sa kok si’; 15, ‘Jin shi’; 16, ‘Huo jing’; 17, ‘Huo shi’; 18, ‘Qu jing shui shi’; 19, ‘Da mo pan’; 20, ‘Hei xin shi’; 21, ‘Mei xian niu xin shi’ (see plant materials).

Table 2. Relevance ratios of the SCAR marker developed from the AFLP marker RO2 in F_1 offspring of ‘Luo tian tian shi’.

Population name (pollen parent)	RO (Okugosho)	RY (Yotsumizo)	RI (Iwasedo)	310 (Taishu)
Number of individuals tested ^z	81	98	49	36
Number of individuals matched ^y	78	93	47	30
Relevance ratio ^x	0.96	0.95	0.96	0.83

^z PCNA/non-PCNA phenotype of each individual was confirmed based on tannin cell size and soluble phenol content.

^y Marker genotype determined by PCR was evaluated for each individual PCNA or non-PCNA phenotype.

^x Ratio of individuals matched in individuals tested.

(Cervantes-Flore et al., 2008a, b; Kriegner et al., 2003). Furthermore, Akagi et al. (2009, 2010) determined the allelic status in the *AST* locus, which contains six *AST/ast* alleles, using quantitative real-time PCR analysis in the hexaploid persimmon. In the future, the three AFLP markers isolated in this study and the above-mentioned new techniques for genetic analysis of polyploid plants would contribute to identification of the *CPCNA* locus in Chinese PCNA persimmon.

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