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Quantitative Genotyping for the Astringency Locus in Hexaploid Persimmon Cultivars using Quantitative Real-time PCR

Takashi Akagi, Yumi Takeda, and Keizo Yonemori[2]
Laboratory of Pomology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Ayako Ikegami
Laboratory of Pomology, Department of Bioproduction Sciences, Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan

Atsushi Kono and Masahiko Yamada[1]
Grape and Persimmon Research Station, National Institute of Fruit Tree Science, Higashi-Hiroshima, Hiroshima 739-2494, Japan

Shinya Kanzaki
Faculty of Agriculture, Kinki University, Nakamachi, Nara 631-8505, Japan

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Abstract. **Persimmon (*Diospyros kaki* Thunb.) is generally hexaploid, and a single *AST* locus controls the pollination-constant non-astringency trait on each of six corresponding chromosomes. The pollination-constant non-astringent (PCNA) genotype is nulliplex and requires homozygous recessive alleles (*ast*) at the *AST* locus. There are several non-PCNA cultivars/selections that could be cross parents; however, the probability of yielding nulliplex offspring depends on the number of recessive alleles (*ast*). In genotyping for the *AST* locus in hexaploid persimmon, in contrast to the situation in diploid plants, we need to detect the *AST*/*ast* allele dosage; this cannot be detected by common codominant markers. In this study, we detected the allele dosage of M_{ast}, which is a marker allele strongly linked to the *ast* allele among cultivars, by quantitative real-time polymerase chain reaction (qPCR) using three reference sites, *actin* (*DkAct*), *anthocyanin reductase* (*DkANR*), and L5R, whose sequences are conserved in the genome of persimmon cultivars. Based on the allele dosage of the M_{ast}, *AST*/*ast* genotypes were estimated for 63 non-astringent cultivars/selections, of which only five cultivars/selections were estimated to be simplex or duplex. The quantitative genotyping method using qPCR may be generally effective for polyploid plants.**

Oriental persimmon, known simply as persimmon, is an important fruit crop in East Asia. Normally, persimmon accumulates abundant condensed tannins (proanthocyanidins) in fruit, which cause strong astringency. One of the main goals of the persimmon industry is to breed superior non-astringent cultivars. The most valuable and preferred cultivar is the pollination-constant non-astringent phenotype, which does not accumulate high amounts of tannins (Akagi et al., 2009a; Yonemori et al., 2000).

The trait of natural astringency loss in pollination-constant non-astringent (PCNA) is controlled by a single gene (*AST*) (Yonemori et al., 2000). Previous reports have suggested that expression of the PCNA genotype requires the *AST* locus to be nulliplex. Indeed, plants that are heterozygous for the *AST* locus will express the astringency trait in fruit flesh (non-PCNA type; Ikeda et al., 1985; Yamada and Sato, 2002). Cultivated persimmon is hexaploid ($2n = 6x = 90$) (Namikawa and Higashi, 1928; Tamura et al., 1998; Zhuang et al., 1990b), and Kanzaki et al. (2001, 2008) suggested the polysomic inheritance of the *AST* locus from the segregation ratio of *AST*-linked marker polymorphisms. Recently, Akagi et al. (2009b) determined the number of marker alleles linked to the *AST* locus using qPCR analysis and showed that six alleles are responsible for the astringency trait in persimmon, demonstrating that PCNA genotype is nulliplex at the *AST* locus.

In the actual breeding of PCNA-type persimmons, the hexaploid inheritance of the *AST*/*ast* allele restricts the breeder in intercrossing PCNA-type cultivars, selections, or both. When some non-PCNA-type cultivars were used as parents, PCNA offspring could only be obtained by a backcross of non-PCNA offspring obtained by a PCNA × non-PCNA cross to a PCNA genotype, and the percentage of PCNA-type offspring was low (Ikeda et al., 1985; Yamada and Sato, 2002). The ineffectiveness of such a cross is the main reason for the avoidance of non-PCNA genotypes in previous breeding programs. At present, the number of available PCNA-type cultivars is limited, and repeated crosses using these cultivars have resulted in inbreeding depression in progeny (Yamada et al., 1994). Hence, a good breeding strategy requires the inclusion of non-PCNA genotypes to widen the genetic pool and to avoid inbreeding depression.

In diploid plants, amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNAs (RAPDs), or simple sequence repeats (SSRs) are commonly used as dominant or codominant markers to detect polymorphisms (D'Surney et al., 2001). Dominant markers only detect the presence or absence of one of the two alleles. Codominant markers can

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[1]Current address: NIFTS, Tsukuba, 305-8605, Japan.
[2]Corresponding author. E-mail: keizo@kais.kyoto-u.ac.jp.

detect the allelic state of both alleles, but are not usually used to quantify the allelic dosage in polyploid plants. In hexaploid persimmon, although a few restricted fragment length polymorphism (RFLP) or sequence-amplified characterized region (SCAR) markers linked to the *AST/ast* genes were identified by Kanzaki et al. (2008, 2009); these markers cannot detect the allelic state at the *AST* locus. In contrast to the situation in diploid plants, the relative ratio of two or more alleles must be determined to infer the genotype and the resulting phenotype of polyploid plants. Quantitative PCR has been used to measure chromosomal dosage for yeast (Bond et al., 2004) and to determine allele dosage in the tetraploid arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] (Henry et al., 2006). In hexaploid persimmon, Akagi et al. (2009b) demonstrated the possibility of determining the *AST*/*ast* allele dosage by qPCR in a well-characterized selection. Information on the *AST*/*ast* allele dosage at the *AST* locus of a non-PCNA parent will greatly contribute to breeding superior PCNAs. If the *ast* allele of a non-PCNA parent is single dose (AAAAAa genotype) or double dose (AAAAaa), no hybridization can yield any PCNA offspring in crosses between non-PCNA and PCNA types because the ratio of the PCNA offspring depends on the ratio of a gamete whose genotype is "aaa," which is derived from a non-PCNA parent (Table 1). When the *ast* allele of a non-PCNA parent is more than triple dose (AAAaaa, AAaaaa, or Aaaaaa), it is possible that crosses between the non-PCNA and PCNA yield PCNA offspring at ≥5% under autoploid or autoalloploid inheritance (Table 1). To date, a few reports and recent unpublished results on some persimmon cultivars suggested the possibility that hexaploid persimmon is basically autohexaploid, but a few cultivars show autoallohexaploid and the details are not fully understood (Akagi et al., 2009b; Akagi and Yonemori, 2008; T. Akagi, unpublished data). The ambiguous inheritance mode is suggested in general polyploidy plants (Comai, 2005). For instance, allotetraploid sour cherry (*Prunus cerasus* L.) derived from hybridization between the diploid sweet cherry (*Prunus avium* L.) and the tetraploid ground cherry (*Prunus fruticosa* L.) shows a complex mode of inheritance in interspecific reciprocal crosses between sweet cherry and ground cherry (Hauck et al., 2006).

Kanzaki et al. (2001, 2008, 2009) identified a polymorphic marker linked to the *AST* locus in two breeding selections. The polymorphisms linked to the *AST* or *ast* alleles in this marker locus were well-characterized and showed four large-scale indels (Fig. 1; Kanzaki et al., 2008; T. Akagi, unpublished data). One of them, the retroposon-like ≈2.5-kb insertion on this marker locus was strongly linked to the *ast* allele (Akagi et al., 2009b; Kanzaki et al., 2009). This marker allele is denoted as M_{ast}, and the other three markers, which are linked to the *AST* allele, are denoted as M_{AST1-3} in this study (see Fig. 1). The objective of the present study is to demonstrate that qPCR analyses to determine the allele dosage of M_{ast} can be used to show the various genotypes of the *AST*/*ast* locus in the 60 non-PCNA cultivars. Information on the *ast* allele dosage or frequency among the cultivars will also provide an insight into the progress of *ast* allele accumulation. Furthermore, we will discuss further application of quantitative genotyping with qPCR not only for the *AST* locus, but also for other loci with polysomic inheritance.

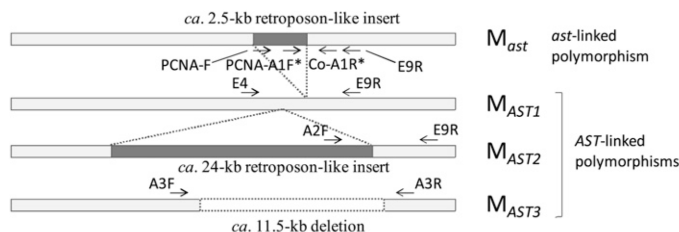

Fig. 1. Large-scale polymorphisms in M_{ast} and M_{AST1-3} (Kanzaki et al., 2009; T. Akagi, unpublished data), and the specific primer sets targeting each polymorphism shown in Table 3 (arrows indicate the primers). Primers with an asterisk were used in quantitative real-time PCR analysis for M_{ast} (Table 3).

Materials and Methods

Identification of an ast-linked marker allele conserved among cultivars. To confirm the validity of our hypothesis that M_{ast} is linked to the *ast* gene but not to the *AST* gene among cultivars, we tested whether non-PCNA cultivars have at least one marker allele for M_{AST}, while PCNA cultivars only have M_{ast}. We used the 60 non-PCNA cultivars shown in Table 2 and the following 23 PCNA cultivars: Fujiwara-gosho, Fukuro-gosho, Fuyu, Gosho, Ikutomi, Izu, Izushi-ogosho, Jiro, Hana-gosho, Kanshu, Maekawa-jiro, Matsumotowase-fuyu, Midai, Mikado, Mushiroda-gosho, Ogosho, Okugosho, Suruga, Soshu, Taishu, Tenjin-gosho, Tokuda-gosho, and Yoshimoto-gosho.

Genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) and was subjected to PCR analyses using the four PCR primers sets specific to the four marker alleles shown in Fig. 1. Briefly, we used primer sets (PCNA-F, E9R) for M_{ast}, (E4, E9R) for M_{AST1}, (A2F, E9R) for M_{AST2}, and (A3F, A3R) for M_{AST3} (see Fig. 1; Kanzaki et al., 2009; T. Akagi, unpublished data). Primer

Table 1. Each genotype at the *AST* locus in non-pollination-constant, nonastringent (non-PCNA) persimmon parents and corresponding ratio of the pollination-constant, nonastringent (PCNA) phenotype in F_1 progeny, in crosses with PCNA types.

Hybridization (non-PCNA genotype) × (PCNA genotype)	*ast* allele dosage of non-PCNA	Ratio of the PCNA phenotype in F_1 (%)[z]	
		Autohexaploid	Autoallohexaploid (genotype in each karyotype of non-PCNA)
AAAAAA × aaaaaa		0	0
AAAAAa × aaaaaa	Single	0	0
AAAAaa × aaaaaa	Double	0	0
AAAaaa × aaaaaa	Triple	5	0 (AAAa/aa or Aaaa/AA), 8.3 (AAaa/Aa)
AAaaaa × aaaaaa	Quadrivalent	20	16.7 (AAaa/aa), 25 (Aaaa/Aa)
Aaaaaa × aaaaaa	Pentavalent	50	50

[z]In hexaploid persimmon cultivars, multivalent chromosome pairing is not exactly observed at meiosis (Zhuang et al., 1990a). We show the segregation ratio without consideration of random chromatid assortment or the possibility of double reduction.

Table 2. Detection of the M_{ast} allele dosage and estimation of the quantitative genotype in the *AST* locus by quantitative real-time PCR analysis in non-pollination-constant nonastringent (non-PCNA) persimmon cultivars.

	Calculated M_{ast} dosage (no. ± SE)[z]			Estimated genotype for the *AST* locus		
Cultivar	*DkAct* reference	*DkANR* reference	L5R reference	*DkAct* reference	*DkANR* reference	L5R reference
Busshigano	4.41 ± 0.46	5.08 ± 0.39	3.68 ± 0.32	Aaaaaa or AAaaaa	Aaaaaa	AAaaaa
Egosho	4.69 ± 0.33	4.42 ± 0.44	4.18 ± 0.48	Aaaaaa or AAaaaa	Aaaaaa or AAaaaa	AAaaaa
Yoshino	4.81 ± 0.37	5.24 ± 0.41	4.29 ± 0.31	Aaaaaa	Aaaaaa	AAaaaa
Akazu	3.20 ± 0.26	3.14 ± 0.23	3.37 ± 0.31	AAAaaa	AAAaaa	AAAaaa
Amahyakume	2.79 ± 0.12	3.27 ± 0.21	2.81 ± 0.17	AAAaaa	AAAaaa	AAAaaa
Kanro	3.54 ± 0.21	3.15 ± 0.15	2.94 ± 0.18	AAaaaa or AAAaaa	AAAaaa	AAAaaa
Kosyu-hyakume	3.15 ± 0.18	2.62 ± 0.26	3.09 ± 0.23	AAAaaa	AAAaaa or AAAAaa	AAAaaa
Kuramitsu	5.18 ± 0.47	3.23 ± 0.37	2.80 ± 0.21	Aaaaaa	AAAaaa	AAAaaa
Kunitomi	3.11 ± 0.21	3.15 ± 0.18	2.83 ± 0.21	AAAaaa	AAAaaa	AAAaaa
Kurokuma	2.77 ± 0.22	2.80 ± 0.29	3.05 ± 0.18	AAAaaa	AAAaaa	AAAaaa
Meotogaki	2.48 ± 0.33	3.34 ± 0.20	2.74 ± 0.24	AAAaaa or AAAAaa	AAAaaa	AAAaaa
Mizushima	2.95 ± 0.17	3.54 ± 0.11	3.23 ± 0.14	AAAaaa	AAaaaa or AAAaaa	AAAaaa
Omidansi	3.12 ± 0.15	3.17 ± 0.22	2.68 ± 0.35	AAAaaa	AAAaaa	AAAaaa
Senbongaki	2.79 ± 0.06	3.39 ± 0.23	3.20 ± 0.16	AAAaaa	AAAaaa	AAAaaa
Tenryubo	2.81 ± 0.16	3.10 ± 0.24	3.00 ± 0.21	AAAaaa	AAAaaa	AAAaaa
Aizu-mishirazu	2.60 ± 0.18	2.09 ± 0.16	1.87 ± 0.29	AAAaaa or AAAAaa	AAAAaa	AAAAaa
Atago	2.10 ± 0.09	2.34 ± 0.21	2.22 ± 0.20	AAAAaa	AAAAaa	AAAAaa
Chong-Do-Si[y]	1.95 ± 0.18	2.33 ± 0.17	1.89 ± 0.20	AAAAaa	AAAAaa	AAAAaa
Dojo-hachiya	2.04 ± 0.24	1.89 ± 0.18	1.94 ± 0.11	AAAAaa	AAAAaa	AAAAaa
Fudegaki	2.89 ± 0.21	2.47 ± 0.29	2.27 ± 0.42	AAAaaa	AAAaaa or AAAAaa	AAAAaa
Gionbo	3.17 ± 0.25	2.40 ± 0.20	1.96 ± 0.17	AAAaaa	AAAAaa	AAAAaa
Joren	2.25 ± 0.13	2.37 ± 0.11	2.02 ± 0.14	AAAAaa	AAAAaa	AAAAaa
Kanzou	3.11 ± 0.14	2.23 ± 0.32	1.83 ± 0.15	AAAaaa	AAAAaa	AAAAaa
Kosyuhachiya	1.90 ± 0.05	1.83 ± 0.23	1.65 ± 0.26	AAAAaa	AAAAaa	AAAAaa
Monpei	2.19 ± 0.06	1.99 ± 0.24	2.11 ± 0.14	AAAAaa	AAAAaa	AAAAaa
Oniwa	1.94 ± 0.29	2.01 ± 0.15	1.71 ± 0.28	AAAAaa	AAAAaa	AAAAaa
Sangokuichi	1.83 ± 0.18	2.08 ± 0.26	1.98 ± 0.19	AAAAaa	AAAAaa	AAAAaa
Seikan	1.92 ± 0.20	1.97 ± 0.18	2.52 ± 0.10	AAAAaa	AAAAaa	AAAaaa or AAAAaa
Shinpei	2.58 ± 0.31	2.24 ± 0.10	2.10 ± 0.12	AAAaaa or AAAAaa	AAAAaa	AAAAaa
Wase-myotan	2.38 ± 0.12	2.60 ± 0.10	2.12 ± 0.16	AAAaaa or AAAAaa	AAAaaa or AAAAaa	AAAAaa
Yashima	1.75 ± 0.18	1.80 ± 0.21	2.09 ± 0.16	AAAAaa	AAAAaa	AAAAaa
Yokono	2.42 ± 0.14	1.78 ± 0.12	2.00 ± 0.26	AAAaaa or AAAAaa	AAAAaa	AAAAaa
Zenjimaru	2.01 ± 0.05	1.74 ± 0.23	2.06 ± 0.21	AAAAaa	AAAAaa	AAAAaa
Akadansi	1.16 ± 0.17	1.42 ± 0.15	1.39 ± 0.18	AAAAAa	AAAAAa	AAAAAa
Ama-yotsumizo	1.05 ± 0.18	1.05 ± 0.06	0.85 ± 0.09	AAAAAa	AAAAAa	AAAAAa
Chokenji	1.20 ± 0.09	0.97 ± 0.07	1.07 ± 0.17	AAAAAa	AAAAAa	AAAAAa
Emon	1.06 ± 0.11	0.93 ± 0.18	1.13 ± 0.11	AAAAAa	AAAAAa	AAAAAa
Hei-xin-shi[x]	0.93 ± 0.21	0.64 ± 0.23	0.79 ± 0.13	AAAAAa	AAAAAa	AAAAAa
Pun kwi Ko Jon-si[y]	0.91 ± 0.02	1.46 ± 0.09	1.06 ± 0.13	AAAAAa	AAAAaa or AAAAAa	AAAAAa
Jisha	0.87 ± 0.13	1.43 ± 0.08	1.08 ± 0.24	AAAAAa	AAAAAa	AAAAAa
Komino	1.50 ± 0.09	1.42 ± 0.12	1.01 ± 0.05	AAAAaa or AAAAAa	AAAAaa or AAAAAa	AAAAAa
Mino	1.04 ± 0.12	1.05 ± 0.13	0.94 ± 0.18	AAAAAa	AAAAAa	AAAAAa
Miyadogaki	1.38 ± 0.14	1.34 ± 0.16	1.11 ± 0.27	AAAAaa or AAAAAa	AAAAAa	AAAAAa
Mopanshi[x]	0.97 ± 0.07	1.04 ± 0.15	1.13 ± 0.16	AAAAAa	AAAAAa	AAAAAa
Nishimurawase	0.90 ± 0.08	0.92 ± 0.16	0.71 ± 0.21	AAAAAa	AAAAAa	AAAAAa
Okumyotan	1.07 ± 0.19	0.94 ± 0.09	0.92 ± 0.12	AAAAAa	AAAAAa	AAAAAa
Onaga	1.00 ± 0.19	1.08 ± 0.21	1.14 ± 0.11	AAAAAa	AAAAAa	AAAAAa
Raotianhong[x]	1.19 ± 0.21	1.02 ± 0.09	0.85 ± 0.17	AAAAAa	AAAAAa	AAAAAa
Saijyo	1.29 ± 0.12	1.15 ± 0.03	1.13 ± 0.06	AAAAAa	AAAAAa	AAAAAa
Shima-myotan	0.98 ± 0.13	1.29 ± 0.09	1.01 ± 0.14	AAAAAa	AAAAAa	AAAAAa
Takura	0.93 ± 0.04	1.09 ± 0.15	0.88 ± 0.11	AAAAAa	AAAAAa	AAAAAa
Yotsuya-saijyo	0.88 ± 0.15	1.01 ± 0.13	0.70 ± 0.06	AAAAAa	AAAAAa	AAAAAa

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Table 2. Continued.

	Calculated M_{ast} dosage (no. ± SE)[z]			Estimated genotype for the *AST* locus		
Cultivar	*DkAct* reference	*DkANR* reference	L5R reference	*DkAct* reference	*DkANR* reference	L5R reference
Akagaki	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	AAAAAA	AAAAAA	AAAAAA
Dennai	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	AAAAAA	AAAAAA	AAAAAA
Hagakushi	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	AAAAAA	AAAAAA	AAAAAA
Luo-tian-tian-shi[w]	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	AAAAAA	AAAAAA	AAAAAA
Mikatanigosho	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	AAAAAA	AAAAAA	AAAAAA
Shogatsu	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	AAAAAA	AAAAAA	AAAAAA
Touhachi	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	AAAAAA	AAAAAA	AAAAAA
To-shi	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	AAAAAA	AAAAAA	AAAAAA
Selection						
275–13	4.12 ± 0.36	3.89 ± 0.12	3.77 ± 0.34	AAaaaa	AAaaaa	AAaaaa
170–26	2.73 ± 0.13	2.88 ± 0.26	3.25 ± 0.29	AAAaaa	AAAaaa	AAAaaa
325–22	4.61 ± 0.25	4.51 ± 0.37	4.98 ± 0.24	Aaaaaa or AAaaaa	Aaaaaa or AAaaaa	Aaaaaa

[z]Calculated M_{ast} dosage is [(relative DNA dosage of M_{ast})/(relative DNA dosage of each reference site)] × 6, based on the fact that 'Jiro' has six M_{ast} alleles in its genome. Relative DNA dosage was calculated from each Ct using the standard curves of the genomic DNA of 'Jiro' shown in Fig. 2.
[y]Korean cultivar.
[x]Chinese cultivar.
[w]'Luo-tian-tian-shi' is a Chinese non-astringent cultivar whose genetic mechanism for the astringency loss is different from that of Japanese pollination-constant, non-astringent (PCNA) cultivars (Ikegami et al., 2006).

sequences are given in Table 3. We analyzed the sequences of the PCR products amplifying the M_{ast}, which is the 2.5-kb inserted fragment *ast*-linked marker allele, using ExoSAP-IT (GE Healthcare Bio-Sciences, Piscataway, NJ) and CEQ8000 (version 7.0; Beckman Coulter, Tokyo).

Examination of the reference sites for qPCR in the persimmon genome. In qPCR analysis to determine the allele dose in the genome, a reference site where the number in the genome and sequences are conserved among the samples is necessary (Henry et al., 2006). Transposable sites, such as transposon- or retroposon-like, or high-copy genes are not suitable because the detected number of the sites could vary among persimmon cultivars. Considering the possibility that there are null or mutated alleles in a reference site locus, we performed three independent qPCR analyses using three reference sites in this study. For the qPCR reference site, we selected the genomic sequence of the two genes encoding actin (*DkAct*) (accession no. AB473616; Akagi et al., 2009a) and anthocyanidin reductase (*DkANR*) (accession no. AB195284; Akagi et al., 2009a; Ikegami et al., 2007), and one genomic marker locus named

Table 3. Primer information for general use in PCR analysis and for quantitative real-time PCR analysis. Each polymorphism of M_{ast} and M_{AST1-3} is shown in Fig. 1.

Marker locus		Primer name	Primer sequences	Tm value[z]	Fragment size (bp)[y]
Primers for general use					
M_{ast}	Forward	PCNA-F	5′- GTGTAATACCCGGTATTAAATAATAAGAG –3′	54.9	ca. 2,500
	Reverse	E9R	5′- GCTTAGTCAGCTTAGCCACGCCATTTC –3′	66.6	
M_{AST1}	Forward	E4	5′- CCCACTTACCAAACTAGGCTTCCAACACAAG –3′	66.3	ca. 2,000
	Reverse	E9R	5′- GCTTAGTCAGCTTAGCCACGCCATTTC –3′	66.6	
M_{AST2}	Forward	A2F	5′- AATCGCAATCCGTCGCAA –3′	59.7	ca. 200
	Reverse	E9R	5′- GCTTAGTCAGCTTAGCCACGCCATTTC –3′	66.6	
M_{AST3}	Forward	A3F	5′- TATTCTGAATATTGGGATAAACGTC –3′	54.6	ca. 350
	Reverse	A3R	5′- GTTCTACACTAGTGAAAATTCCACAA –3′	54.8	
Primers for quantitative real-time PCR					
M_{ast}	Forward	PCNA-A1-F	5′- CCTGAGAATTTCCTTATAGTGACACG –3′	58.4	100
	Reverse	Co-A1-R	5′- ATTTCGCGGGATGCCTCTA –3′	59.2	
DkAct	Forward	DkAct-RTF	5′- CATGGAGAAAATCTGGCATCATAC –3′	58.5	71
	Reverse	DkAct-RTR	5′- GAAGCACTGGGTGCTCTTCTG –3′	58.7	
DkANR	Forward	DkANR-RTF1	5′- TGCCCATGTGGAGGATGTTT –3′	59.8	78
	Reverse	DkANR-RTR1	5′- CAGCAAATGTACCGACCAGAAG –3′	58.4	
L5R	Forward	Consv-5F	5′- TCTGGAACGATGCTCTTGCA –3′	59.1	80
	Reverse	Consv-5R	5′- TGAAGATCAACGGCAAGTCCT –3′	58.4	

[z]Tm value was calculated using Primer Express Software (version 2.1; Applied Biosystems, Tokyo).
[y]Lengths of the amplified fragment in 'Kuramitsu' are shown.

L5R (accession no. AB537429; T. Akagi, unpublished data), the sequence of which is conserved in the genus *Diospyros* L. and is linked to the *AST* locus. To identify the conserved sequences of *DkAct*, *DkANR*, and L5R, their PCR products were directly analyzed using ExoSAP-IT and CEQ8000 (version 7.0). The conserved sequences were used as reference sites for qPCR. The primers for qPCR were designed using Primer Express Software (version 2.1; Applied Biosystems, Tokyo).

qPCR analysis for determination of the M_{ast} allelic dosage. We used the 60 non-PCNA cultivars shown in Table 2 to determine the M_{ast} allele dosage using qPCR analysis. In addition to these cultivars, we used three breeding selections, 170–26, 275–13, and 325–22, whose genotypes at the *AST* locus have been suggested from the segregation ratio of their offspring (Kanzaki et al., 2001, 2007, 2008; Yamada and Sato, 2002). DNA samples were thoroughly purified with phenol and chloroform extraction and were diluted to a concentration of 60 ng·μL^{-1} to keep a constant amplification efficiency among samples in the following qPCR analysis. Samples were analyzed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems) with the SYBR Green system using SYBR Premix Ex Taq (TaKaRa, Tokyo). All reactions were carried out in a total volume of 25 μL/well, consisting of 12.5 μL of SYBR Premix, 9 μL of sterilized distilled water, 1 μL of each of forward and reverse detection primers (5 μM), 0.5 μL of Rox dye, and 1 μL of template DNA. All DNA samples were completely denatured at 95 °C in a heating block for 10 min and were then cooled on ice before each reaction. The standard amplification protocol consisted of an initial denaturing step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 15 s, 57 °C for 5 s, and 72 °C for 15 s. The primer sequences used for detection of M_{ast}, *DkANR*, and L5R are shown in Table 3.

Standard curves for the M_{ast} and two reference sites were generated using two replications of eight serial 2-fold dilutions of genomic DNA of 'Jiro' (PCNA), which has six M_{ast} in its genome (Akagi et al., 2009b). We set the maximum DNA concentration of the standard sample at approximately 120 ng·μL^{-1}. In the qPCR analysis, the average threshold cycle (*Ct*) was automatically determined by ABI PRISM 7900HT as the default state. *Ct* is defined as the point at which fluorescence rises appreciably above the background. For each measurement, independent standard curves were constructed and three replications of each sample were analyzed. The mean C_t of three replications was used for each sample. The detected value of the M_{ast} relative to that of the reference site represents the M_{ast} allelic dosage in the genome relative to that of the standard sample 'Jiro'.

Results

M_{ast} is strongly linked to the ast gene in persimmon cultivars. All 25 PCNA cultivars had only M_{ast} among the four marker alleles shown in Fig. 1 (data not shown). In contrast, all 60 non-PCNA cultivars had at least one of the other three marker alleles, M_{AST1}, M_{AST2}, and M_{AST3} putatively linked to the *AST* gene (Fig. 1), and most of them also had M_{ast}. These results indicate that only M_{ast} is linked to the *ast* gene among the four alleles in the cultivars, and that non-PCNA cultivars possibly have many *ast* genes. The sequence analysis for M_{ast} showed single nucleotide polymorphisms among the cultivars. We designed the primer sets (forward: PCNA-A1F and reverse: Co-A1R) by amplifying the M_{ast} fragment specifically using the conserved sequences among the cultivars (Table 3).

qPCR analysis estimation of the ast allele dosage. We designed three specific primer sets for reference site of qPCR analysis, (DkActRTF and DkActRTR) for *DkAct*, (DkANR-RTF1 and DkANR-RTR1) for *DkANR*, and (Consv-5F and Consv-5R) for L5R, using the conserved sequences among the cultivars (Table 3). In qPCR analysis, the amplification efficiency is an important factor affecting the results. The ideal value of the amplification efficiency is 100%, which means the quantity of amplified fragments is doubled after one thermal cycle of PCR reaction. In this qPCR analysis, the amplification efficiencies of the *DkAct*, *DkANR*, L5R, and M_{ast} markers were on average 95.1% ± 1.2%, 96.4% ± 0.6%, 96.6% ± 1.3%, and 97.6% ± 2.7%, respectively, and all standard curves were significantly linear (P > 0.995) (Fig. 2, the standard curves generated from genomic DNA of 'Jiro'). These were valid enough to allow analysis of the allelic dosage in the genome.

The M_{ast} allelic dosage relative to that of the standard sample 'Jiro' was determined using qPCR analyses. We calculated the number of M_{ast} alleles in the genome based on the fact that 'Jiro' has six M_{ast} alleles (Akagi et al., 2009b). Our qPCR

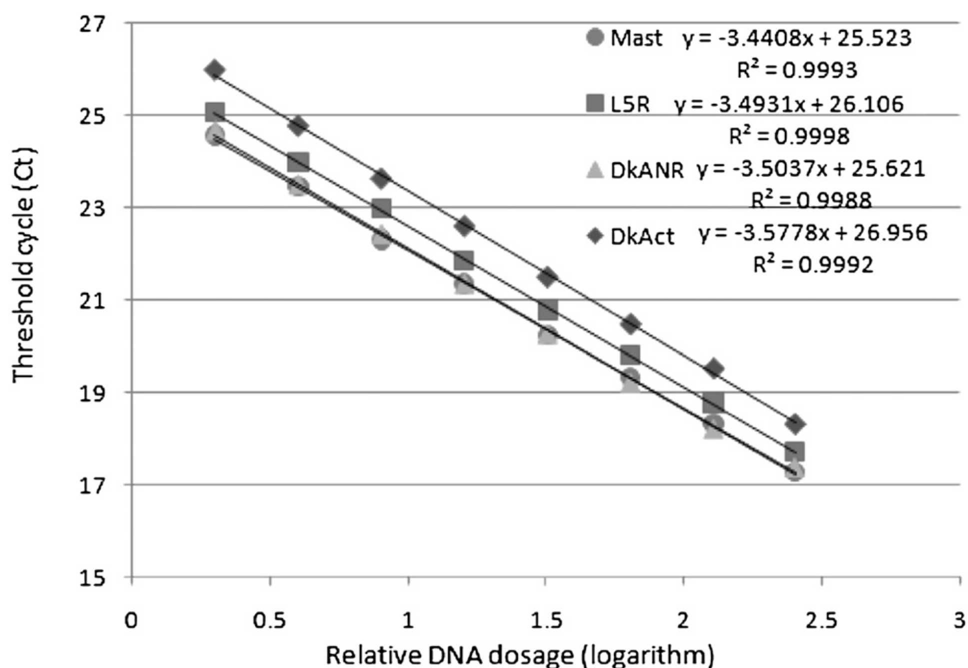

Fig. 2. Standard curves for M_{ast} (round points), L5R (square points), *DkANR* (triangular points), and *DkAct* (diamond-shaped points) in quantitative real-time PCR analyses using eight serial two-fold dilutions of the genomic DNA of 'Jiro' (PCNA). The x-axis indicates threshold cycle (Ct), automatically determined by ABI7900HT as the default state. The y-axis indicates relative DNA dosage expressed as logarithms. The minimum relative DNA dosage at the highest dilution of the genomic DNA of 'Jiro' was defined as "1."

results accurately showed the number of M_{ast} alleles in the genome and the estimated genotypes at the *AST* locus in the 60 non-PCNA cultivars and three breeding selections (Table 2). The number of M_{ast} or the estimated genotype at the *AST* locus was generally consistent in the three independent calculations using *DkAct*, *DkANR*, or L5R as reference sites. This result supported the validity of the three reference sites for qPCR analysis of the persimmon genome. Only 'Kuramitsu' showed strikingly different M_{ast} dosage or estimated genotypes, and a few cultivars had slighter inconsistencies, with the three reference sites (Table 2). This is possibly due to some mutated or null alleles in the reference sites that reduce amplification in qPCR analysis. To avoid this problem, several reference sites are needed for qPCR analysis when genotyping persimmon cultivars.

Most non-PCNA cultivars had zero to three M_{ast}, indicating an estimated genotype of AAAAAA, AAAAAa, AAAAaa, or AAAaaa at the *AST* locus (Table 2). The ratio of each estimated genotype at the *AST* locus among the 60 non-PCNA cultivars was as follows: 14.3% (AAAAAA), 33.9% (AAAAAa), 32.1% (AAAAaa), 21.4% (AAAaaa), and 5.4% (AAaaaa or Aaaaaa). The average of the M_{ast} allele dosage in a cultivar ranged from 1.72 to 1.77. The estimated *ast* allelic frequency in the 60 cultivars was 29%. Previous reports by Kanzaki et al. (2001, 2007, 2008) and Yamada and Sato (2002) suggested the *AST*/*ast* genotypes of three breeding selections, 170–26, 275–13, and 325–22, from the segregation ratio of their offspring in the *AST*/*ast* (non-PCNA/PCNA) phenotypes. The dominant *AST* allele dosage of 170–26, 275–13, and 325–22 was suggested to be triple, double, and single dose, which indicate the *AST*/*ast* genotypes of AAAaaa, AAaaaa, and Aaaaaa, respectively. Our results showed that the estimated genotypes of 170–26, 275–13, and 325–22 are AAAaaa, AAaaaa, and Aaaaaa, respectively (Table 2), which are consistent with the results from the segregation analyses.

The *AST*/*ast* genotypes of 'Busshigano', 'Egosho', and 'Yoshino' were estimated to be Aaaaaa or AAaaaa (Table 2). In addition, 12 cultivars were estimated to have the triple-dose *ast* alleles (Table 2). At present, when a non-PCNA-type cultivar is used as a parent, PCNA offspring are obtained only by backcrossing the second generation, and the ratio of obtainable PCNA offspring is low (Ikeda et al., 1985; Yamada and Sato, 2002). However, our results suggest that some non-PCNA cultivars have a triple dose or more of the *ast* allele, meaning that crosses between them and a PCNA genotype would yield about 5% to 50% PCNA offspring under autohexaploid inheritance in a single generation.

Discussion

Examination and application of the methodology of qPCR analysis for quantitative genotyping. Quantitative PCR to determine the gene or allele dosage was first used in the field of medicine (Ochshorn et al., 2006; Zimmermann et al., 2002) because changes in gene dosage cause significant damage to humans. Plant molecular biologists applied qPCR to determine the copy number of a transgene (Song et al., 2002) and to study karyotypes in arabidopsis (Henry et al., 2006). Henry et al. (2006) also proposed the novel strategy of genotyping for polyploid plants by quantitative analysis of allele dosage with qPCR. In our study, we applied this qPCR methodology to quantitative genotyping of the *AST* locus in several hexaploid persimmon cultivars. We accurately estimated the *AST*/*ast* genotype in almost all of the non-PCNA cultivars studied (Table 2).

However, our methodology for the quantitative *AST*/*ast* genotyping by qPCR still has a few problems. There were significant calculation errors in the measurements for cultivars that had a high *ast* allele dosage, such as Busshigano, Egosho, and Yoshino (Table 2, see SE). This is presumably due to the methodology of the qPCR analysis. When we compare the single-dose allele to double-dose allele by qPCR analysis, a 2-fold difference is detected. When we compare the quadrivalent-dose allele to pentavalent-dose allele, the ability to detect a 1.25-fold difference is required. That is difficult and accompanies some errors in analysis because the detection of a 1.25-fold difference means detection of a difference of about 0.322 (= $\log_2 1.25$) cycles in real-time PCR analysis. A solution to this problem is to measure the number of marker alleles linked to the dominant *AST* gene because the more the recessive *ast* alleles are, the less the dominant *AST* alleles are in a genome. The detection of dominant and recessive alleles by qPCR will enable exact quantitative genotyping. However, in this study, we could not detect the dosage of M_{AST} linked to the dominant *AST* allele because of the M_{AST} polymorphisms shown in Fig. 1. It is necessary to identify the conserved sequences linked to the *AST* allele or the *AST* gene itself to detect the dosage of the *AST* allele.

Quantitative genotyping by qPCR is effective in two respects, marker-assisted selection (MAS) and the genome mapping of polyploid plants. Quantitative genotyping by qPCR could contribute to MAS, especially for recessive traits in polyploid plants, such as the PCNA trait in persimmon, because the probability of obtaining nulliplex offspring greatly depends on the dosage of the recessive allele in the parents, an example of which is shown in Table 1. We can also apply quantitative genotyping for loci whose allele balances involve quantitative traits in polyploid plants. Furthermore, genome-wide mapping of autohexaploid sweetpotato [*Ipomoea batatas* (L.) Lam.] has been reported recently (Cervantes-Flores et al., 2008a, 2008b; Kriegner et al., 2003). In these reports, the segregation ratio of offspring was modeled corresponding to quantitative genotype in each marker locus. Allele dosage or quantitative genotyping in each marker locus is important in this mapping. Hence, the qPCR approach for quantitative genotyping would be possibly applicable to this genome-wide mapping in autoploid plants. To date, a number of maps in well-characterized autopolyploid plants, such as sugarcane (*Saccharum* L.) or potato (*Solanum tuberosum* L.), have been developed using various approaches (e.g., interspecific crossing or comparative genomic analysis) (Garcia et al., 2006; Le Cunff et al., 2008; Ming et al., 2002), but genome-wide mapping of other many autopolyploid plants has not progressed yet mainly due to various technical difficulties derived from genomic complexity (Henry et al., 2006; Luo et al., 2006). The qPCR method for quantitative genotyping could be one of the simple solutions to this problem and would facilitate the genome mapping in many autopolyploid plants, including persimmon.

Transition of ast allelic frequency possibly elucidates the origin of PCNA type. Our results suggested that quantitative *AST*/*ast* genotypes vary among the 60 non-PCNA cultivars (Table 2). The *ast* allelic frequency was 29%. The cultivar with the highest *ast* allele dosage, Yoshino, originates from the same area as a PCNA cultivar, Gosho. In addition,

'Egosho' and 'Busshigano', which also contain a high *ast* allele dosage (Table 2), originate in areas near to that of 'Gosho' or other major PCNA cultivars such as Fuyu. The existence of 'Gosho' is first mentioned in the seventeenth century (Kikuchi, 1948), and 'Gosho' was the only PCNA cultivar to be grown over a wide area 200 years ago (Yamada, 2005). This suggests that 'Gosho' is the original PCNA, as inferred by Yamada (2005), and that the accumulation of the *ast* allele and the resultant generation of the PCNA phenotype occurred around the region where 'Gosho' arose. There are more than 1500 cultivars of persimmon in China, Japan, Korea, and Europe (Wang et al., 1997; Yamada, 2005). The *ast* allelic frequency is presumed to differ among countries. Therefore, a detailed detection of the *ast* allelic frequency among the cultivar groups in these areas could contribute to understanding how the *ast* allele has accumulated.

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