A New *S*-allele in Apple, 'Sg', and Its Similarity to the 'Sf' Allele from 'Fuji'

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Abstract. *S*-allele genotypes of nine apple (*Malus ×domestica* Borkh.) cultivars were identified using *S*-allele-specific polymerase chain reaction (PCR)-restriction fragment-length polymorphism (RFLP) analysis. A new *S*-allele, *Sg*, was proposed to be present in 'American Summer Pearmain', 'Indo', 'Kitanosachi', and 'Meku 10'. This allele is very similar to *Sf* at the nucleotide sequence (92%) and deduced amino acid sequence (94%) levels.

Apple exhibits gametophytic self-incompatibility controlled by a single multiallelic locus, the *S*-locus (de Nettancourt, 1977). Pollen tube growth is inhibited when the *S*-allele carried by the pollen matches one of the two *S*-alleles expressed in the pistil. Knowledge of the *S*-genotype of apple cultivars is useful in selecting compatible parents in a breeding program. The *S2*, *S3*, *S5*, *S7*, *S9* (= *Sc* at deduced amino acid level), and *Sf* genes of apple cultivars have been sequenced by Broothaerts et al. (1995), Janssens et al. (1995), and Sassa et al. (1996), and an *S*-allele-specific PCR analysis developed by Janssens et al. (1995), and modified by Sakurai et al. (1997) and Matsumoto et al. (1999) is a useful method for *S*-allele identification.

In this study, we investigated *S*-allele genotypes of nine apple cultivars by *S*-allele-specific PCR-RFLP analysis. We identified a new *S*-allele, '*Sg*', which is remarkably similar to *Sf*, and documented its presence in 'American Summer Pearmain', 'Indo', 'Kitanosachi', and 'Meku 10'.

Materials and Methods

Plant material. Most plants used in this study were from collections at the Apple Research Center of the National Institute of Fruit Tree Science in Morioka, Japan. 'Shinano

Sweet' and 'Chouka 15' were obtained from the Nagano Fruit Tree Experiment Station. Young leaves were collected and stored at -80 °C until use.

***S*-allele-specific PCR analysis of DNA.** Total DNA from leaves of individual plants was isolated as described by Thomas et al. (1993). *S2*-, *S3*-, *S7*-, and *Sf*-allele-specific PCR amplification and digestion were carried out essentially as described by Matsumoto et al. (1999). For *Sg* allele identification, PCR was conducted using sense (5'-attaatctgctcgacttg-3'; *Sf*-sense 2 in Fig. 1) and antisense (5'-ttggtgggcagaaattcc-3'; *Sf*-antisense 2 in Fig. 1) primers, with the total DNA of each cultivar being expected to possess the *Sf* or *Sg* allele. The following cultivars served as templates: 'Alps Otome', 'American Summer Pearmain', 'Chouka 15', 'Indo', 'Kitanosachi', 'Meku 10', 'Senshu', and 'Shinano Sweet'. Each 35 µL PCR mixture contained 1.0 µM of each primer, 200 µM deoxynucleotides, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, and 2.5 U *Taq* polymerase, and 50 ng template DNA. The analysis was programmed in a thermal cycler (GeneAmp 2400 apparatus; Perkin-Elmer Co., Urayasu, Chiba, Japan) and conducted under the following conditions: 3 min at 94 °C for preheating; 1 min at 94 °C, 1 min at 56 or 62 °C, and 1 min at 72 °C for 30 cycles; followed by an extension for 10 min at 72 °C. After PCR, the amplified fragments were digested by *Rsa*I or *Sau*3AI. The

amplified and digested fragments were separated on 1% agarose, 4% polyacrylamide, or 8% polyacrylamide gels in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 50–70 V for 30 to 90 min.

***S*-allele sequence analyses of 'Indo' and 'Fuji' genomic DNA.** PCR was performed using the sense and the antisense primers described above, with 'Fuji' or 'Indo' DNA. Conditions for PCR were as described above. The sequences of electroeluted PCR products were determined directly by dideoxy chain termination on an ABI PRISM™ 377 DNA sequencer (Perkin-Elmer Co.) using ABI PRISM™ Cycle Sequencing kits (Perkin-Elmer Co.).

Results and Discussion

We identified the *S2*-, *S3*-, *S7*-, *S9*-, and *Sf*-alleles of 'Alps Otome', 'American Summer Pearmain', 'Chouka 15', 'Indo', 'Kitanosachi', 'Meku 10', 'Senshu', 'Shinano Sweet', and 'Toukou' using the *S*-allele-specific PCR-RFLP analysis method described by Matsumoto et al. (1999) (Table 1). The *S*-allele genotypes of 'Indo' and 'Kitanosachi' were initially identified as *S7Sf*. Although crosses of 'Indo' × 'Kitanosachi' and 'Senshu' (*S7Sf*) × 'Iwakami' (*S7Sf*) were incompatible, 'Indo' × 'Senshu' and 'Indo' × 'Iwakami' were compatible (100% and 96% of fruit set, respectively). As the *S7*-allele of 'Senshu' was inherited from 'Indo' via 'Toukou', the *S7* allele of 'Indo' and 'Senshu' should be the same. However, the *Sf* alleles 'Senshu' and 'Iwakami' ('Fuji' × 'Jonathan') were inherited from 'Fuji', but those of 'Indo' and 'Kitanosachi' were not. As the *Sf* gene was isolated from 'Fuji', 'Senshu' and 'Iwakami' might contain the same *Sf* gene as 'Fuji'. We investigated whether or not the identified *Sf*-allele within 'Indo' is exactly the same as that of 'Fuji'. In Fig. 1, the genomic sequence of the amplified fragment using the *Sf* allele-specific primers, *Sf*-sense 2 and *Sf*-antisense 2, and with 'Indo' DNA, were slightly different from that of 'Fuji' (92% similarity). The product amplified by *Sf*-sense and *Sf*-antisense primers is primarily used for *Sf*-allele identification. Since this region is quite similar to the corresponding region of 'Indo', 'Indo' might have been misclassified as having the *S7Sf* allele (Fig. 1). We propose that the primary identified *Sf* allele within 'Indo' be designated '*Sg*'.

From the sequence data of *Sg* ('Indo') and *Sf* ('Fuji'), a method to distinguish *Sg* and *Sf*

Table 1. *S*-allele genotypes of apple cultivars.

Cultivars	Parentage	<i>S</i> -allele genotypes
Alps Otome	Unknown	<i>S9Sf</i>
American Summer Pearmain	Unknown	<i>SfSg</i>
Chouka 15	Golden Delicious (<i>S2S3</i>) ² × Senshu	<i>S3Sf</i>
Indo	Unknown	<i>S7Sg</i>
Kitanosachi	Tsugaru (<i>S3S7</i>) ² × American Summer Pearmain	<i>S7Sg</i>
Meku 10	Tsugaru (<i>S3S7</i>) ² × American Summer Pearmain	<i>S3Sg</i>
Senshu	Toukou × Fuji (<i>S9Sf</i>) ²	<i>S7Sf</i>
Shinano Sweet	Fuji (<i>S9Sf</i>) ² × Tsugaru (<i>S3S7</i>) ²	<i>S7Sf</i>
Toukou	Golden Delicious (<i>S2S3</i>) ² × Indo	<i>S2S7</i>

²Data on *S*-allele genotypes from Matsumoto et al. (1999).

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<i>Sg</i>	AAATAATATTATTCAAATGGGACGGGATTATATATATCG	60
<i>Sf</i>	ATTAATCTGCCGCGCACTTG.....C.....T.....G.....G.....	
	<i>Sf-sense 2</i> <i>Sf-sense</i>	
<i>Sg</i>	TTATGATGGTATTTTCACTAATTTTATTAATATTGCTCTCCGACGGTGGGATTGATT	120
<i>Sf</i>		
<i>Sg</i>	ATTATCAATTTACCGAGCAATATCAGCCGCTCTTGTGCACTCTAATCCAACTCTCTGTA	180
<i>Sf</i>	A.....	
<i>Sg</i>	AGGATCTCTCTGACAAGTTGTTTACTGTTACGGGTTGTGGCTTCAGACTCGAATCGAA	240
<i>Sf</i>	C.....T.....A.....	
<i>Sg</i>	ATCACCACAAATATTGCAAGGCGCGGCATATCAGACGGTAATATTATTAGCATAATCAG	300
<i>Sf</i>	G.....T.....A.....T.....	
	<i>Sf-antisense 2</i>	
<i>Sg</i>	ATAGTCAATATTCTTTATCTCAATTTATCTACCTCTCTGTGT--ATATATATATTTTGGGA	360
<i>Sf</i>	A.....C.....GT.....	
<i>Sg</i>	TATGCTAGACCCCAAAATTTTAAACCAATGATG--CAT--TATA--TATGATAC	420
<i>Sf</i>	A.....T.....C.....T.....TGT.....CAG.....AAA.....AC.....	
<i>Sg</i>	CTAATTAACACTTAAGTAATAATTCATCATCAACATCTACATAATTTCTTACAAAAA	480
<i>Sf</i>	T.....C.....A.....C.....A.....	
<i>Sg</i>	TTTAATCTTACTATCATTTACTCTATATTTTTTGATATATACATATAGTCAACACAGCT-	540
<i>Sf</i>	G.....TG.....C.....G.....T.....C.....T.....	
<i>Sg</i>	---A---CA---A---A---TATTACAAATTAATTTAAATTTAATCATAAATTTATTCGG	600
<i>Sf</i>	TTC-TG---GGCGTGT-G-AA.....TA	
<i>Sg</i>	TTATATATTATTATATTGTCTAGATAAAATACTCGAACACAGTTGGCAATTATTGGCC	660
<i>Sf</i>	G.....C.....T.....	
<i>Sg</i>	GAACCTACTTCAATCGAAACATCATGGAAGGCTTCTGGCGTAAACAGTGGGAGAAACATGG	720
<i>Sf</i>	T.....	
<i>Sg</i>	CTCCCTGTGGGTCTTCCCCANTACAGAACAGCATTACTTTTGATACGTAAATCAAAAT	780
<i>Sf</i>	T.....	
<i>Sg</i>	GTACACAACCCAGAAACAAACGCTCTCTCAANTCTCTCAAAAGGCCANTATAAAACCGGC	840
<i>Sf</i>	T.....	
<i>Sg</i>	TAGCAAAAACAGGCACTGCTCCATATTCCAAATGCCATGCCAATTTATCAACAATAT	900
<i>Sf</i>	C.....C.....	
<i>Sg</i>	GACACCAAAATTCAGTGCACAAAGAAATACTAGGACATCATTTGACTGAATTGGTTGAGGT	960
<i>Sf</i>		
<i>Sg</i>	CGCTCTTTTCACCCATACCAACTTAAGCAATTCATAAATTCGCGCGGCCATTTCACCA	1020
<i>Sf</i>	T.....T.....	
<i>Sg</i>	AGGATCTAC-----	1028
<i>Sf</i>	GCAATTTCTGCCCCACCAA	1047
	<i>Sf-antisense 2</i>	

Fig. 1. Nucleotide sequences of the 'Indo' (*Sg*) and 'Fuji' (*Sf*) genomic sequences amplified by using *Sf*-sense 2 and *Sf*-antisense 2 primers. Nucleotides common to the *Sg* and *Sf* are presented by dots, whereas nucleotide changes are indicated by uppercase letters. The *Sf*-sense 2, *Sf*-sense, *Sf*-antisense 2, and *Sf*-antisense primer regions are identified. *Sau*3AI and *Rsa*I sites are boxed with normal and bold lines, respectively. The underlined nucleotide sequences represent consensus sequences of plant 5' and 3' splice sites. The 'Indo' sequences corresponding to the *Sf*-sense 2 and *Sf*-antisense 2 primers are not yet known and are indicated as dashed lines.

using restriction enzyme analysis was developed. By *Sau*3AI digestion, 471, 343, 182, and 26 bp fragments were obtained from the 1022 bp genomic fragments of *Sg* ('Indo'), while 497, 343, 182, and 26 bp fragments were obtained from 1048 bp of *Sf* ('Fuji') (Fig. 1). Moreover, by *Rsa*I digestion, 329, 266, 122, 116, 111, and 78 bp fragments should be from *Sg* ('Indo'), and 337, 329, 266, and 116 bp fragments should be from *Sf* ('Fuji') (Fig. 1). In this case, the 337 bp fragment of *Sf* ('Fuji') should be observed as 122, 111, and 78 bp in *Sg* ('Indo') because of the additional two *Rsa*I sites present in *Sg* ('Indo') (Fig. 1). After the *Sau*3AI or *Rsa*I digestion, all of the fragments detected in 'Senshu' matched the expected fragments of *Sf* ('Fuji') (Fig. 2), suggesting that 'Senshu' contains the *Sf* allele (Table 1). The fragment patterns of 'Alps Otome', 'Chouka 15', and 'Shinano Sweet' also completely matched that expected with *Sf* ('Fuji'), suggesting that they also contain the *Sf* allele (Table 1). Yet, 'Kitanosachi' showed identical fragment patterns with that expected of *Sg* ('Indo') (Fig. 2). As 'Meku 10' also showed identical fragment patterns with *Sg* ('Indo'), both 'Kitanosachi' and 'Meku 10' might contain the *Sg* gene (Table 1). Since 'American Summer Pearmain', the pollen parent of 'Kitanosachi' and 'Meku 10', should contain *Sg*, and mixed fragment patterns of *Sg* and *Sf* were obtained from it after *Sau*3AI and *Rsa*I digestion (Fig. 2), it was identified as having the genotype *SfSg* (Table 1).

Sg contains one intron deduced from the presence of plant 5' and 3' splice-site consensus sequences comparable in size (318 bp in *Sg*, 344 bp in *Sf*) and location to that of the *Sf* allele within 'Fuji' (Sassa et al., 1996). The AT content of the intron is very high (78%), which is consistent with plant intron recognition (Haseloff et al., 1997). Only 12 of 216 amino acids differed between the *Sg* and *Sf* deduced sequences of the sequenced region (Fig. 3). No differences were observed in the five short stretches of highly conserved regions (C1, C2, C3, C4, and C5) within the *S*-alleles. Kao and McCubbin (1996) showed that hypervariable (HV) regions (HVa and HVb) were necessary but not sufficient for *S*-allele specificity in *Petunia inflata* Fries, but that HV regions alone were sufficient to confer *S*-allele specificity in *Solanum chacoense* Bitt. (Matton et al., 1997). Of 12 amino acids, two were present in the HVa region, and 10 were outside the HV regions (Fig. 3). More research is needed on the site(s) related to *S*-allele specificity.

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Fig. 2. *Sg*- and *Sf*-allele-specific PCR-RFLP analysis. PCR products obtained by using 'Senshu' (*S7Sf*) (lanes 2, 5, 6), 'Kitanosachi' (*S7Sg*) (lanes 3, 7, 8) and 'American Summer Pearmain' (*SfSg*) (lanes 4, 9, 10) genomic DNA as templates, and *Sf*-sense 2 and *Sf*-antisense 2 primer were digested by *Sau*3AI (lanes 2–4) or *Rsa*I (lanes 5, 7, 9 on 4% polyacrylamide gel; lanes 6, 8, 10 on 8% polyacrylamide gel). Lanes 1 and 11 are size markers on 4% and 8% polyacrylamide gels, respectively.

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Fig. 3. Deduced amino acid sequences of the 'Indo' (*Sg*) and 'Fuji' (*Sf*) genomic sequences of Fig. 1. Completely conserved residues between *Sg* and *Sf* are presented by dots whereas amino acid changes are indicated. The conserved regions C1 through to C5, and hypervariable regions HVa and HVb are boxed. The last 10 amino acids of *Sf* are from Sassa et al. (1996).