Sequence of the S_{10} cDNA from ‘McIntosh’ Apple and a PCR-digestion Identification Method

Kentaro Kitahara

The United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan

Shogo Matsumoto¹

Department of Biology, Faculty of Education, Gifu University, Gifu 501-1193, Japan

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Abstract. An S -allele cDNA was cloned from pistils of ‘McIntosh’ apple (*Malus × domestica* Borkh.). The allele, designated S_i in Japan and S_{10} in Europe, is an S -RNase that is very similar (94%) to the S_3 -RNase at the deduced amino acid sequence level. This allele can be detected by amplification using the polymerase chain reaction (PCR) and specific primers, followed by digestion with restriction enzyme *EheI*. The S_{10} allele was discovered in ‘Empire’, ‘Maypole’, ‘Shinano Red’, ‘Spencer’, and ‘Vista Bella’. The S -allele cDNAs sequenced to date are listed with their Japanese and European designations.

Apple exhibits gametophytic self-incompatibility (GSI). GSI is controlled by a single locus, the S -locus. In this type of self-incompatibility, pollen tube growth is arrested in the style when the pollen shares the same S -allele with the pistil (de Nettancourt, 1977).

The S -alleles in the pistil encode ribonucleases, called S -RNases. To date, six S -RNases (S_c , S_d , S_e , S_f , S_g , and S_h) have been cloned in Japan (Kitahara et al., 1999, 2000; Matsumoto and Kitahara, 2000; Sassa et al., 1996), and eight S -RNases (S_2 , S_3 , S_5 , S_7 , S_9 , S_{24} , S_{26} , and S_{27}) in Europe (Broothaerts et al., 1995; Janssens et al., 1995; Verdoodt et al., 1998). Two pairs of S -RNases, S_c and S_g , and S_h and S_{24} , are identical at the deduced amino acid sequence level.

Recently, the S -alleles of ‘McIntosh’ were identified as S_{25} by pollination and progeny analysis (Komori et al., 1999), and as $S_{10}S_{25}$ by the stylar ribonuclease bands (Bošković and Tobutt, 1999). In the present study, the cDNA sequence encoding the S_i -RNase was determined; the same allele is designated S_{10} in Europe. We developed a S_i ($=S_{10}$)-allele-specific PCR-digestion analysis method, and determined that S_i ($=S_{10}$) is present in ‘McIntosh’ and five other cultivars.

Materials and Methods

Plant material. Leaves of ‘Empire’, ‘Shinano Red’, ‘Spencer’, and ‘Vista Bella’ were obtained from the Nagano Fruit Tree Experiment Station, Japan. Other *Malus* plants used in this study were from collections at the Apple Research Center of the National Institute of Fruit Tree Science, Morioka, Japan. ‘McIntosh’ flowers were collected at the popcorn stage and dissected, and the pistils were removed and immediately frozen in liquid nitrogen and stored at -80°C . Young leaves were collected and stored at -80°C until used.

S_i -RNase cDNA cloning. Total RNAs of the mature pistils without the ovule, stamens, petals, and leaves from ‘McIntosh’ were extracted essentially as described by Chang et al. (1993). Reverse transcription (RT) was performed in a total volume of 20 μL containing 1 μL (1.0 μg) of RNA, 0.125 μM Oligo dT-Adaptor Primer (Takara Shuzo Co., Ltd., Kusatsu, Shiga, Japan), 0.25 U AMV Reverse Transcriptase XL (Takara Shuzo Co., Ltd.), 20 U RNase Inhibitor (Takara Shuzo Co., Ltd.), 1 mM dNTPs, 5 mM MgCl_2 , and 1x RNA PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl). The analysis was programmed in a thermal cycler (GeneAmp 2400 apparatus; Perkin Elmer Co., Ltd., Urayasu, Chiba, Japan) and conducted under the following conditions: 60 min at 42°C , 5 min at 99°C , and cooling to 4°C . A PCR was then carried out in a total volume of 100 μL containing 20 μL of the RT reaction mixture, 0.2 μM OWB134 primer (Broothaerts et al., 1995), 0.2 μM M13 Primer M4 (Takara Shuzo Co., Ltd.), 2.5 mM MgCl_2 , 1x RNA PCR buffer, and 2.5 U *Taq* DNA polymerase (TaKaRa *Taq*TM; Takara Shuzo Co., Ltd.). The OWB134 primer anneals to a conserved region in apple S -RNases and the M4 primer is complementary to the adaptor

region of the OligodT-Adaptor primer. The amplification program consisted of 2 min at 94°C for initial denaturation, 45 cycles of 30 s at 94°C , 30 s at 50°C , 1.5 min at 72°C , and a final extension of 7 min at 72°C .

The cDNAs obtained were subcloned into a pCR2.1 cloning vector with the TA cloning kits (Invitrogen Co., Ltd., Hongo bunkyo-ku, Tokyo). The nucleotide sequences of four clones designated Si-1, -2, -3, and -4 were determined by dideoxy chain termination on an ABI PRISMTM 377 DNA sequencer (Perkin-Elmer Co., Ltd.) using dRhodamine Terminator Cycle Sequencing Kits (Perkin-Elmer Co., Ltd.).

S -allele specific PCR analysis of *Malus × domestica* DNA. Total DNA from leaves of individual plants was isolated as described by Thomas et al. (1993). S -allele-specific PCR amplification and digestion were carried out essentially as described by Broothaerts et al. (1995), Kitahara et al. (2000), Matsumoto and Kitahara (2000), and Verdoodt et al. (1998). For S_i ($=S_{10}$)-allele identification, PCR was conducted using sense (5'-AACAAATCTTAAAGCCAGC-3'; Si-sense, nucleotides 255–274 in Fig. 1) and antisense (5'-GGTTTCTTATAGTCGATACTTTG-3'; Si-antisense, nucleotides 514–536 in Fig. 1) primers, with the total DNA of each cultivar being expected to possess the S_i ($=S_{10}$)- or S_j -allele. 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 MgCl_2 , 0.001% (W/V) gelatin, 2.5 U *Taq* polymerase, and 50 ng template DNA. The thermal cycler (GeneAmp 2400 apparatus; Perkin Elmer Co., Ltd.) was programmed for the following conditions: 3 min preheating at 94°C , 1 min at 94°C , 1 min at 60°C , and 1 min at 72°C for 30 cycles, followed by an extension for 10 min at 72°C . After PCR, *EheI* digested the amplified fragments. Digestion was done for 2 h at 37°C . The amplified and digested fragments were separated on a 4% polyacrylamide gel in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 50–70 V for 1–1.5 h.

Results

Since the OWB134 primer anneals to a conserved region in apple S -RNases, we used it for cloning the S -RNases in ‘McIntosh’ (Verdoodt et al., 1998). We isolated and sequenced four independent cDNA clones, and all inserts represented a single S -allele, designated the S_i -RNase. We did not obtain the cDNA clone corresponding to the other S -allele in ‘McIntosh’ by using the OWB134 primer. The 3'-nucleotide triplet of the OWB134 primer corresponds with the translation initiation codon ATG (Verdoodt et al., 1998). The S_i -RNase contains a 684 bp coding region encoding 228 amino acids with a MW and pI value of 26121 and 9.42, respectively (Fig. 1). In apples, S -RNases code for polypeptides containing 225 to 228 amino acids. A part of the protein sequence of S_i -RNase (deduced amino acid residues 28 to 210 in Fig. 1) completely matched that of the S_{10} -RNase

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¹To whom reprint requests should be addressed. Phone: +81-58-293-2257; fax: +81-58-293-2207; e-mail: shmatsum@cc.gifu-u.ac.jp

(Richman et al., 1997), suggesting that S_1 -RNase is equivalent to S_{10} -RNase. The deduced amino acid sequences of S_1 -RNase showed a high similarity to S_3 -RNase (94%).

From the S_1 -RNase cDNA sequence data, we developed a PCR-digestion method for S_i ($=S_{10}$)-allele identification. We designed specific primers, 'Si-sense' and 'Si-antisense', and carried out PCR amplification using 'McIntosh' as a template. A PCR product very close to 282 bp was obtained for 'McIntosh' (Fig. 2, lane 1) and 'Golden Delicious' (lane 3), but not from five other cultivars (lanes 5–9). Digestion of the 282 bp fragment from 'McIntosh' by *EheI* cleaved it into pieces of 185 bp and 97 bp (Fig. 2, lane 2), but the fragment from 'Golden Delicious' was not digested (Fig. 2, lane 4). The amplified allele in 'Golden Delicious' (S_{23}) must be S_3 , as no fragment was amplified in 'Worcester Pearmain' (S_{24}). S_3 is the only other S -allele that is amplified by the primers. Using this method, the presence of the S_i ($=S_{10}$)-allele was also detected in 'Empire', 'Spencer', 'Maypole', 'Vista Bella', and 'Shinano Red' (Table 1). The S -genotypes of 'Empire' and 'Spencer' are consistent with their parentage, as both are seedlings of 'McIntosh'. The second allele in these cultivars (S_2 , S_3 , S_{24} , S_{27} , and S_{30}) was identified using the S -allele specific PCR-digestion analysis method (Table 1).

Discussion

In this study, we isolated a cDNA clone of the S_1 -RNase from 'McIntosh' apple. Only 14 out of 228 amino acids differed between the S_1 and S_3 protein sequences. Of these 14 differences, two were in the rosaceous hypervariable (RHV) region. Several unclear bands were obtained at high molecular weight by the primers 'FTQYQ' and 'anti- 1_M WPNV' (Matsumoto and Kitahara, 2000). As the sequences of the primers completely match corresponding regions of the S_1 -RNase, a band of 1300 bp seemed to be amplified from the S_1 -RNase. If this interpretation is correct, the S_1 -RNase might contain an intron at a similar position and length as the S_3 -RNase. Since S_1 and S_3 -RNase are closely related, as are S_7 , S_8 , and S_{10} -RNases, they might be useful to investigate the relationships among S -alleles in apple.

It was clarified that the S_1 -RNase corresponds to the S_{10} -RNase. In Japan, S -alleles have been designated alphabetically (Komori et al., 1994, 1998a, 1998b, 2000), but numerically in Europe (Kobel et al., 1939). Kitahara et al. (2000) listed several alleles by their alphabetical designations and the corresponding numerical designation. Table 2 includes additional equivalent designations and lists cultivars that carry each allele (Broothaerts et al., 1996; Sakurai et al., 1997). The accession numbers of the DNA sequences are also shown (Table 2). Recently, we cloned S_6 -RNase from 'Delicious' (Matsumoto and Kitahara, 2000). A part of the deduced amino acid sequence (amino acid residues 71 to 189) completely matches that of the S_{30} allele of 'Red Delicious' (GenBank data base Accession number

ATGGGGATTACAGGGATGATATATATGGTTACAATGGTATTTTCATTAATTTGTAATTAATG	60
M G I T G M I Y M V T M V F S L I V L M	20
TTGTCTTCGTCCGCGGTGAATTCGATTATTTTCAATTTACGCAGCAATATCAGCCGGCT	120
L S S S A V K F D Y F Q F T Q Q Y Q P A	40
GTCGCAACTCTAATCCTACTCCTTGTAAAGGATCCTCCGGACAAGTTGTTACGGTTCAT	180
V C N S N P T P C K D P P D K L F T V H	60
GGTTTGTGGCCTTCAATGTTAATGGAAGTGACCCCAAGAAATGCAAAGCTACATCTTA	240
G L W P S N V N G S D P K K C K A T I L	80
AATCCTCAAAAGATAACAAATCTTAAAGCCAGCTGGAAATTAATTTGGCCAAACGTACTC	300
N P Q T I T N L K A Q L E I I W P N V L	100
AATCGAAGGGCTCATGTACGCTTCTGGCGTAAACAGTGGCGTAAACATGGCGCCTGTGGG	360
N R R A H V R F W R K Q W R K H G A C G	120
TACCCACAAATAGCGGACGACATGCATTACTTTAGCACAGTAATCGAAATGTACATAACC	420
Y P T I A D D M H Y F S T V I E M Y I T	140
AAGAAACAAACGCTCTCTGAAATCCTCTCAAAGGCGAAGATTAAACCGGAGGGGAGATTTC	480
K K Q N V S E I L S K A K I K P E G R F	160
AGGACACGGGACGACATTGTAAATGCCATAAGCCAAAGTATCGACTATAAGAAACCCAAA	540
R T R D D I V N A I S Q S I D Y K K P K	180
CTCAAGTGCAAGATCAATAATCAGACAACTGAATTGGTTGAGGTTCGGTCTTTGCAGCGAT	600
L K C K I N N Q T T E L V E V G L C S D	200
AACAACTTAACGCAGTTCATAAATTGCCCCAACCCATTTCCTCAAGGATCACCATATTTTC	660
N N L T Q F I N C P N P F P Q G S P Y F	220
TGCCCCACCAATAATATTTCAGTATTAAGAGCGCAGCTAGATAGTATTGCATATACGGATG	720
C P T N N I Q Y *	228
ATGATTTTCATATATAGGACGAGAGGAATGCAGTTATGTGATTGTATTCTAATGAATAA	780
ATTGTCTTAATTAATTAAACTAGTGTGAATATGTGAATTTTCCTTTCACGTGAACCTTGG	840
CCTATATACGGATATTATAATGTAGAATAAAAAAATAAACTTGATCAGTAAAAA	900
AAAAA	905

Fig. 1 Nucleotide and deduced amino acid sequences of the S_1 -RNase cDNA from 'McIntosh' apple. The first three nucleotides were from the 3'-nucleotide triplet of the OWB 134 primer. The asterisk indicates the stop codon. The sequence of the S_1 -RNase cDNA was deposited under the DDBJ accession number AB052683.

Fig. 2 S_1 ($=S_{10}$)-allele specific PCR-digestion analysis. PCR products obtained from 'McIntosh' ($S_{10}S_{25}$) (lane 1,2), 'Golden Delicious' (S_2S_3) (lane 3,4), 'Samsa' (S_2S_7) (lane 5), 'Delicious' (S_9S_{30}) (lane 6), 'American Summer Pearmain' (S_7S_{20}) (lane 7), 'Worcester Pearmain' (S_2S_{24}) (lane 8), and 'Baskatong' ($S_{29}S_{27}$) (lane 9) genomic DNA as templates, and 'Si-sense' and 'Si-antisense' primers. The products of lane 1 and 3 were digested by *EheI* (lanes 2,4).

AF201748, released 1 Dec. 2000). The amino acid residues 1 to 70 and 190 to 227 are not listed in the database for S_{30} , so the degree of match with S_e is unknown. The matched region (71 to 189) contains a RHV region, which distinguishes among S-RNases. The intron position and sequence of the S_{30} -RNase also completely match those of S_e -RNase (Accession number AB050636 submitted by us). Similarly, a part of the amino acid sequence of the S_1 -RNase (Cortal et al., 1999) completely matches amino acid residues 41 to 90, including the RHV region of S_e -RNase (Matsumoto et al., 1999b; Sassa et al., 1996), suggesting that S_e corresponds to S_1 . Previously, we represented the S -genotype of 'Mutsu' as $S_{\alpha}S_{\beta}S_{\gamma}$ (Kitahara et al., 1999), while it was reported as $S_2S_3S_{20}$ by Bošković and Tobutt (1999). From these results, S_{γ} corresponds to S_{20} . Bošković and Tobutt (1999) designated the S -genotype of 'McIntosh' as $S_{10}S_{25}$. Since S_1 corresponds to S_{10} , S_{25} should correspond to S_2 , although the cDNA of this allele has not yet been cloned and sequenced.

Sakurai et al. (2000) identified S_d in 'Delicious' and its progeny 'Empire', 'Melrose', 'Murasaki', 'NY75413-30', 'NY79507-72', 'Orei', and 'Toyo' by PCR analysis following Katoh et al. (1997). But, as we pointed out previously (Komori et al., 1994; Matsumoto and Kitahara, 2000), S_e ($=S_{30}$) has priority over S_d and should be used for 'Delicious' and its progeny. 'Cameo', 'Ginger Gold', 'Golden Supreme', 'Gold Rush', and 'Korei' also carry the S_e ($=S_{30}$)-allele. The S -allele genotypes of 'Hokuto', 'Jonathan', and 'Mutsu' were listed as $S_eS_eS_f$, S_eS_{α} , and $S_{\alpha}S_{\beta}S_{\gamma}$, respectively, from two-dimensional gel electrophoresis profiles of stylar S -glycoprotein (Sassa et al., 1994). The S -allele in 'Hokuto' and 'Jonathan' designated S_e by Sassa et al. (1994) differs from that in 'Delicious' (Matsumoto et al., 1999a; Sakurai et al., 2000). This allele of 'Hokuto' and 'Jonathan' should be designated S_d ($=S_7$) (Komori et al., 1994), and the S_d -allele of 'Mutsu' should be changed to S_{γ} ($=S_{20}$). Alternatively, the European numerical designations can be used without confusion (Table 2).

We identified the S_i ($=S_{10}$)-allele in six cultivars (Table 1). The S_i ($=S_{10}$)-allele of 'Empire' and 'Spencer' were inherited from 'McIntosh', and that of 'Shinano Red' from 'Vista Bella'. 'Wijcik' arose as a bud mutation of 'McIntosh' and would be expected to have the same S -alleles (Greene et al., 1997). 'Maypole' ($S_{27}S_i = S_{27}S_{10}$) inherited S_i ($=S_{10}$) from 'Wijcik'. The S_i ($=S_{10}$)-allele was not present in 'Tohoku 2' ('McIntosh' x 'Worcester Pearmain') or 'Kitakami' ('Tohoku 2' x 'Red gold'). Since S_{24} and S_9 allele were identified from 'Tohoku 2' and 'Kitakami', respectively, these cultivars should have the S -allele genotypes of $S_{24}S_{25}$ ('Tohoku 2') and S_9S_{25} ('Kitakami').

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Table 1. S -allele genotype of apple cultivars.

Cultivar	Parentage	S -allele genotype	
		European	Japanese
Empire	McIntosh x Delicious	$S_{30}S_{10}$	S_eS_i
Maypole	Wijcik x Baskatong	$S_{27}S_{10}$	$S_{27}S_i$
McIntosh		$S_{10}S_{25}$	$S_{\alpha}S_{\gamma}$
Shinano Red	Tsugaru x Vista Bella	S_3S_{10}	S_8S_i
Spencer	McIntosh x Golden Delicious	$S_{25}S_{10}$	$S_{\alpha}S_i$
Vista Bella		$S_{24}S_{10}$	S_hS_i

Table 2. S -alleles of major apple cultivars and equivalent Japanese and European designations.

Allele Designation		Cultivar	S -alleles	Database Accession #	Reference
European	Japanese				
1	f	Ralls Janet	1 2		Matsumoto et al., 1999a
		Northern Spy	1 3		Sakurai et al., 2000
		Senshu	1 7		Matsumoto et al., 1999b
		Hokuto	1 7 9		Sakurai et al., 2000
		Fuji ^z	1 9	D50837	Sassa et al., 1996
2	a	Golden Delicious ^z	2 3	U12199	Broothaerts et al., 1995
		Jonagold	2 3 9		Broothaerts et al., 1996
		Orin	2 7		Sakurai et al., 1997
		Mutsu	2 3 20		Kitahara et al., 1999
3	b	Golden Delicious ^z	2 3	U12200	Broothaerts et al., 1995
		Tsugaru	3 7		Matsumoto et al., 1999a
5	-	Queen's Cox ^z	5 9	U19791	Janssens et al., 1995
		Cox's Orange Pippin	5 9		Kitahara et al., 2000
7	d	Idared ^z	3 7	U19792	Janssens et al., 1995
		Jonathan	7 9		Janssens et al., 1995
		Akane ^z	7 24	AB032246	Kitahara et al., 2000
9	c	Fuji ^z	1 9	D50836	Sassa et al., 1996
		Queen's Cox ^z	5 9	U19793	Janssens et al., 1995
10	i	McIntosh ^z	10 25	AB052683	this paper
20	g	Indo ^z	7 20	AB019184	Kitahara et al., 1999
24	h	Akane ^z	7 24	AB032247	Kitahara et al., 2000
		Braeburn ^z	9 24	AF016920	Verdoodt et al., 1998
25	z	McIntosh	10 25	not yet sequenced	
26	-	Baskatong ^z	26 27	AF016918	Verdoodt et al., 1998
27	-	Baskatong ^z	26 27	AF016919	Verdoodt et al., 1998
30	e	Delicious ^z	9 30	AB035273	Matsumoto and Kitahara, 2000

^z S-RNases were isolated from these cultivars.

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