Identification and characterization of *S-RNase* genes in apple rootstock and the diversity of *S-RNases* in *Malus* species

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Abstract We isolated and confirmed two *S-RNases*, denoted as *mpS1* and *mpS2*, from apple rootstock ‘Marubakaido’ (*Malus prunifolia* Borkh. Var. *ringo* Asami). These *S-RNases* contained and conserved five cysteine residues and two histidine residues, which are essential for RNase activity. The *mpS1* showed high similarity to *S5* (99.1%) of *Malus spectabilis*, whereas the *mpS2* showed 99.5% nucleotide sequence similarity to *S26* of (*Malus* × *domestica*) and 99.6% to *S35* of (*Malus sieversii*) when compared with reported *S-RNases*. In amino acid sequences, the *mpS1-RNase* was almost similar to the *S5-RNase* of *Malus spectabilis*, and the *mpS2-RNase* was similar to the *S35* of *Malus sieversii*, with only one bp being different from the *S26-RNase* of *Malus* × *domestica*. The 57 *S-RNases* of *Malus* species were renamed and rearranged containing the new *S-RNases*, as *mprpS35* (*mpS2*) and *mprpS57* (*mpS1*), for determining *S*-genotypes and identifying new alleles from apple species (*Malus* spp.).

Keywords Apple, *Malus prunifolia*, Marubakaido, *S-RNase*, Self-incompatibility

Introduction

Self-incompatibility (SI) is the inability of the pollen grain to

fertilize its own egg in flowering plants. The SI in apple is controlled by the *S*-locus with multiple alleles derived from series of mutation of that particular locus. When the genotype of an *S*-allele of pollen grain is same with that of the pistil, then the pollen grain is recognized as ‘self’ and therefore, the pollen tube fail to grow inside the style. Normally, a ribonuclease known as *S-RNase* encoded by *S*-allele regulates the self-incompatibility in flowering plants (de Nettancourt 1977, 2001).

Apple (*Malus* spp.) shows gametophytic self-incompatibility (GSI) that prevents inbreeding and enhance out crossing. Thereby, artificial pollination is pre-requisite for ensuring fruit set using cross-compatible pollen from other cultivars, including crab apples. This has been extensively conducted in commercial fruit production of apple. Identification of the *S*-genotypes is thus essential for selecting suitable pollen donors in commercial fruit production, and also to initiate breeding programs (Matsumoto 2014).

The *S-RNases* (*S1* to *S29*) and *S*-genotypes of 300 apple cultivars (*Malus* × *domestica*) were determined by pollination test (Kobel et al., 1939; Komori et al. 1999, 2000), protein analyses (Sassa et al. 1994, 1996) and DNA analysis (sequencing, *S*-allele specific PCR and PCR-CAPS; Broothaerts et al. 1995; Janssens et al. 1995; Sassa et al. 1996; Verdoodt et al. 1998; Matsumoto et al. 1999a, b, 2000, 2001a, b; Kitahara et al. 2000; Matsumoto and Kitahara 2000; Schneider et al. 2001; Van Nerum et al. 2001; Kitahara and Matsumoto 2002a, b; Matityahu et al. 2005; Kim et al. 2006; Gu et al. 2015). However, the *S30-RNase* was designed from *St-RNase* of *Malus transitoria* (Matsumoto et al. 2000). Furthermore, two *S-RNases*, *S31* and *S32*, were identified from apple (*M.* × *domestica*) cultivars ‘York Imperial’ and ‘Burgundy’, respectively and arranged the 32 *S*-allele numbers of apple for designing the *S*-allele number of new *S-RNase* of *M.* × *domestica* (Kim et al. 2008).

Recently, new *S-RNases* were also identified from other *Malus* species such as *Malus orientalis* (*Ssi5*), *Malus sieversii*

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(*Ssi5*) and *Malus sylvestris* (*Sad5*) by Matsumoto et al. (2010). The *Ssi5* and *Sad5* were renamed as *S33* and *S34*, respectively to use of the next available numbers. Dreesen et al. (2010) reported that 16 novel *S-RNases* (*S1'*, *S5'*, *S9'*, *S23'*, *S36*, *S37*, *S38*, *S39*, *S40*, *S41*, *S42*, *S43*, *S44*, *S45*, *S46* and *S47*) were identified in European wild apple (*Malus sylvestris*) and the *S*-genotype of 196 *M. sylvestris* trees and 27 *M. sylvestris* - *M. × domestica* hybrid was determined by using PCR based method. In addition, two different *S-RNase* PCR products were obtained from genomic DNA of *Malus spectabilis* cv. 'Haitang No.3' (*MsS3/MsS4*) and the full-length sequences of *MsS3-RNase* were determined (Heng et al. 2011).

'Marubakaido' (*Malus prunifolia* Borkh. Var. *ringo* Asami), which is mutant of *Malus Prunifolia*, widely distributed in northern China and Siberia. That is one of the commonly used native apple rootstock in several countries. This rootstock has advantage of resistant to water logging and humid soil conditions, disease tolerant and ease of propagation by hardwood cuttings. Furthermore, it has also been used as a pollen donor of apple cultivars (*Malus × domestica*) (Inomata et al. 2004, Dantas et al. 2005). Thus, determination of 'Marubakaido' *S*-genotype is important for stable fruit production and efficient breeding of new rootstock.

In this study, we examined the *S*-genotypes of the apple rootstock 'Marubakaido' with the previously described *S-RNase* designed primers and sequence analysis, and identified another two *S-RNases*. So far, too many self-incompatibility genes were identified from various *Malus* species, which are always confusing for their nomenclature. Therefore, it is suggested to rearrange the assigned *S-RNase* number of *Malus* species deploy our newly identified two *S-RNase* in the registered *S-RNase* GenBank.

Materials and Methods

Plant materials

Young leaves of apple cultivar 'Marubakaido' (GeneBank No. JP173432) were collected at the Apple Research Station, NARO Institute of Fruit Tree Science (Morioka, Japan). The leaves were drayed frozen in liquid nitrogen and stored at -80°C.

DNA extraction and PCR amplification

Total genomic DNA was extracted from the young leaves by using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany)

following the manufacturer's instructions with slight modifications (Yamamoto et al. 2006). We previously designed a new primers pair, ASPF3 (5'-CAATTTACGAGCARTATCAG-3') and ASPR3S (5'-CAAAGASHGACCTCAACYAAT TS-3') and developed PCR-CAPS systems for determination of *S*-genotypes and discrimination of 25 *S-RNases* of apple (Kim et al. 2009) and PCR conditions were as described Kim et al. (2006).

Cloning and Sequence analysis of *S-RNases*

Purified PCR products were cloned with TOPO Cloning Kit (Invitrogen, Germany). DNA sequences were determined by using the DNA sequencer (ABI, 3130 Genetic Analyzer). Nucleotide and amino acid sequences were analyzed with the BLAST, CLUSTALW, and Genious 4.8.2 programs to search for homology. Restriction enzyme sites were searched by using MIKENORA RESEARCH Ver. 4.0.

Results and Discussion

Nucleotide sequences of newly identified *S-RNase* genes in 'Marubakaido'

We amplified the *S-RNase* RCR fragments ca. 600 bp from apple rootstock 'Marubakaido' (*Malus prunifolia*) using the common primers (ASPF3 and ASPR3S) which also previously used to amplify the 22 *S-RNases* of (*Malus × domestica*; Kim et al. 2009). Based on the lengths of the amplified fragments, the amplified PCR fragments belong to group I was 618–674 bp including 12 *S-RNases* (*S2*, *S4*, *S6b*, *S7*, *S9*, *S11*, *S21*, *S23*, *S26*, *S28*, *S32* and *Skb*) in four groups of 22 *S-RNases* (Kim et al. 2009).

Thereby, the *S*-genotypes could not be confirmed only by PCR analysis, so we performed cloning and sequencing of the *S-RNase* PCR fragments. Where, we have identified two novel *S-RNase* sequences that contain two exons and one intron, positioned between two exons at hypervariable region (RHV). The sequences are named as *mpS1-RNase* (*Malus prunifolia* S1) and *mpS2-RNase* (*Malus prunifolia* S2) and entered into DNA Data Bank of Japan (DDBJ) with accession numbers AB618796 (*mpS1*) and AB618797 (*mpS2*) (Fig. 1). The *mpS1-RNase* fragment was 644 bp with expected 147 bp intron started at positions 154 bp and continued up to 300 bp (Fig. 1A). The 644 bp nucleotide sequence can be translated to 165 amino acid sequences. Whether, the *mpS2-RNase* is 657 bp long with a 157 bp intron started at the position 160 bp and continued up to 316 bp (Fig. 1B). Thereby, this 657 bp

Fig. 1 Nucleotide and amino acid sequences of *mpS1-RNase* (a) and *mpS2-RNase* (b) of ‘Marubakaidou’ (*Malus prunifolia* var. *ringo*). Underlines indicate the primer site. Italic letters indicate the intron region. Box indicates the *BpmI* site; C/TGGAG, 396+248 (*S32*-specific enzyme of *Malus × domestica*) (A) and the *BsmI* site; GAATG/C, 222+435 (*S26*-specific enzyme of *Malus × domestica*) (B)

nucleotide sequence can be translated to 166 amino acids. Furthermore, the *mpS1-RNase* and *S5-RNase* (*Malus spectabilis*) have similar enzyme site of *BpmI* for *S32*-allele specificity, however both of those *RNase* had low homology (80.7%). The *mpS1* could be distinguished with the *S5-RNase* (*Malus spectabilis*) for two bases difference, but *mpS2* had only one

base difference with *S26-RNase* (*Malus × domestica*) at sequence of intron region (Fig. 2A, B). The restriction enzyme indicated the *BpmI* site; C/TGGAG, 396 + 248 bp (*S32*-specific enzyme of *Malus × domestica*; Fig. 1A) and the *BsmI* site; GAATG/C, 222 + 435 bp (*S26*-specific enzyme of *Malus × domestica*; Fig. 1B).

Comparison of the nucleotide and amino acid sequences of the *mpS1*- and *mpS2-RNase* with those of the 12 *S-RNases* of apple

The similarity between two novel *S-RNases* with registered *S-RNases* of apple (*Malus* spp.) in GenBank was analyzed based on the nucleotide sequences. The *mpS1-RNase* revealed high homology with *S5* (99.1%) of *Malus spectabilis*, *S22* (93.5%) of *Pyrus sinkiangensis*, *Sh* (93.5%) of *P. communis*, *S4* (92.6%) of (*Malus × domestica*), *S21* (92.3%) of *P. bretschneideri* and *S32* (80.7%) of *M. domestica* (Fig. 2A, Table 1). Whereas, the *mpS2-RNase* showed 99.6% homology to *S35* of *Malus sieversii*, 99.5% to *S26* of *Malus × domestica*, 96.2% to *S30* of *Pyrus pyrifolia* and *S30* of *P. ussuriensis*, 94.3% to *S9* of *P. korschinskyi* and 85.4% to *S26* of *P. bretschneideri* (Fig. 2B, Table 1).

The predicted amino acid sequences of two novel *S-RNases* were compared with 12 other apple *S-RNases* revealed and confirmed 5 conserved domains (C1, C2, C3, RC4, and C5) with one hypervariable (HV) region at recognition sites of *S*-allele, that is discriminate for self-incompatible reactions (Ishimizu et al. 1998a, b; Ushijima et al. 1998; Vieira et al. 2007; Fig. 3). The two novel *S-RNases* contain five cysteine and two histidine as conserved residues, which might be essential for structural and functional roles of ribonucleases (Ishimizu et al. 1998a, b). Whereas, the RC4 region was also remained, that is important domain in rosaceous species, because no other homologues sequences were found in T2/S type *RNases*. Therefore, it is suggested that the RC4 domain arisen during divergence of the rosaceous families and

Table 1 Analysis of nucleotide sequence homology among the *S-RNases* of Marubakaidou and Pyrinae

	<i>S5-RNase</i> <i>Malus</i> <i>spectabilis</i>	<i>S22-RNase</i> <i>Pyrus</i> <i>sinkiangensis</i>	<i>Sh-RNase</i> <i>Pyrus</i> <i>communis</i>	<i>S4-RNase</i> <i>Malus ×</i> <i>domestica</i>	<i>S21-RNase</i> <i>Pyrus</i> <i>bretschneideri</i>	<i>S32-RNase</i> <i>Malus</i> <i>domestica</i>
<i>mpS1-RNase</i> ‘Marubakaidou’	99.1%	93.5%	93.5%	92.6%	92.3%	80.7%
	<i>S35-RNase</i> <i>Malus</i> <i>sieversii</i>	<i>S26-RNase</i> <i>Malus ×</i> <i>domestica</i>	<i>S30-RNase</i> <i>Pyrus</i> <i>pyrifolia</i>	<i>S30-RNase</i> <i>Pyrus</i> <i>ussuriensis</i>	<i>S9-RNase</i> <i>Pyrus</i> <i>korshinskyi</i>	<i>S26-RNase</i> <i>Pyrus</i> <i>bretschneideri</i>
<i>mpS2-RNase</i> ‘Marubakaidou’	99.6%	99.5%	96.2%	96.2%	94.3%	85.4%

Fig. 2 Nucleotide sequence alignment of *mpS1*- and *mpS2*-*RNase* of ‘Marubakaidou’ with eight homologous *S*-*RNases* selected on the basis of sequence similarity, using basic local alignment search tool (BLAST). The *mpS1*-*RNase* of ‘Marubakaidou’ shows high the similarity with *S5*-*RNase* of *Malus spectabilis*, but has two different sequences in the intron region, as shown in the boxes (A). The *mpS2*-*RNase* of ‘Marubakaidou’ shows a high similarity with *S35*-*RNase* of *Malus sieversii* and *S26*-*RNase* of *Malus* × *domestica*. To compare with *S26*-*RNase* of *Malus* × *domestica* the *mpS2*-*RNase* has three different sequences, two sequences in the exon and one sequence in the intron are shown in the boxes (B). Arrows indicate the intron region

subfamilies (Ushijima et al. 1998).

Moreover, the *mpS1* and *S5*-*RNase* of *Malus spectabilis* had the similarity in amino acid alignment except at primer site, and the *mpS2* showed the same amino-acid sequences

with *S35*-*RNase* (*Malus sieversii*) but had difference in only one sequence with *S26*-*RNase* of *Malus* × *domestica* (Fig. 3).

Deduced amino acid sequence of *S8*-*RNase* of *Pyrus pyrifolia* ‘Meigetsu’ had high similarity with *S28*-*RNase* of *P.*

Fig. 3 Multiple amino acid sequence alignment of the *mpS1*- and *mpS2*-RNase of Marbakaidou with 12 homologous *S*-RNase of Pyrinae. Almost all the *S*-RNases share the five cysteine residues characteristic of *S*-RNase, and two histidine residues essential for RNase. The putative hypervariable region (*HV*) defined by Ishimizu et al. (1998) and Ushijima et al. (1998) and five conserved regions (C1, C2, C3, RC4 and C5) are underlined

sinkiangensis ‘Kuerlexiangli’ and *S3*-RNase of *Malus spectabilis* ‘Haitang No.3’ showed 100% and 96.9% identity, respectively. However, the nucleotide sequence of intron in *PpS8*-RNase showed highly polymorphic but 95.3% similarity with *PsS28*-RNase and 91.9% homology with *MsS3*-RNase (Heng et al. 2011). Furthermore, pollen tube growth in styles and *S*-haplotypes of *F*₁ plants among *PpS8*-RNase with *PsS28*-RNase and *MsS3*-RNase were segregated including *PpS8PsS28* and *PpS8MsS3*. Heng et al. (2011) suggested that the *PpS8*-RNase, *PsS28*-RNase or *MsS3*-RNase might be intermediate steps to become a new *S*-haplotype without loss of self-incompatibility. In fact, an *S*-haplotype could be unchanged up to a small number of amino acid changes in the *S*-RNase. However, sometimes a minor change might be broadening its specificity. Thus, we decided that the *mpS2* of ‘Marbakaidou’ and *S35*-RNase of *Malus sieversii* are different *S*-allele with the *S26*-RNase of *Malus* × *domestica* but the *mpS1* and *S5*-RNase (*Malus spectabilis*), and the *mpS2* and *S35*-RNase (*Malus sieversii*) are same *S*-allele group with *S57* and *S35*, respectively (Table 2).

Rearrangement of the registered *S*-RNase genes including *mpS1*- and *mpS2*-RNase in apple

There are 25 to 30 species and several subspecies are found in genus *Malus*, which has called crab apples, many of them are cultivated as ornamental trees. They are very attractive for

their profuse blossom and colorful fruits (Janick et al. 1996). The cultivated apple has been developed through interspecific hybridization of different *Malus* spp. Thus, the appropriate scientific name of apple has been accepted as the binomial *Malus* × *domestica* (Korban and Skirvin 1984).

The registered *S*-alleles of apple in the GenBank of NCBI are reviewed for assigning the *S*-allele numbers for two newly identified *S*-RNases. Broothaerts (2003) showed that *S6* and *S19* correspond to *S25* and *S28*, respectively. However, their genomic sequences are partially different. Matsumoto et al. (2003) reported that the sequences of *S6* and *S12*; *S17* and *S19*; *S13* and *S14* are the same. Thereby, those *S*-RNases have now been re-numbered as *S6a*, *S6b*, and *S11*, respectively. Although, *S21*-RNase seemed to correspond to *St*-RNase according PCR-RFLP analysis, but their amino acid sequences showed slight difference, however it also included as *S30* in re-numbering. Matsumoto and Furusawa (2005) have sequenced the genomic DNA for *S16c* (=16)-RNase in ‘Bohnappel’ (*S6bS9S16c*) and have been re-numbered as *S16* (=S27a) in ‘Baskatong’ (*S16*=27aS26) and *S22* (=27b) in ‘Alkmene’ (*S5S22*=27b) as *S16a* and *S16b*, respectively. However, the *S15*- and *S18*-alleles were reported by Bošković and Tobutt (1999), but not yet registered in GenBank.

Recently, three *S*-RNase alleles *S44* from *Malus domestica* and *S45* and *S46* from *M. souldardii* were identified by Long et al. (2010), but later of the same year, 12 new *S*-RNase alleles

Table 2 Newly arranged *S*-alleles number including two newly identified *S*-alleles for *Malus* spp

	<i>Malus domestica</i> Pumilae (Section)	<i>M. sylvestris</i> Pumilae	<i>M. sieversii</i> Pumilae	<i>M. angustifolia</i> Chloromeles	<i>M. kansuensis</i> Sorbomalus	<i>M. prunifolia</i> Pumilae
			<i>M. transitoria</i> Sorbomalus	<i>M. mandshurica</i> Gymnomeles	<i>M. spectabilis</i> Pumilae	
S1	mdopS1(Sf(D50837))	msypS1 (S1'(EU419866))				
S2	mdopS2(S2(U12199), Sa, S33(DQ219464), S37(EU391607), S38(EU391609), S39(EU3916010), S40(EU391611), S41(EU391612))					
S3	mdopS3(S3(U12200), Sb)					
S4	mdopS4(S4(AF327223))					
S5	mdopS5(S5(U197991))	msypS5 (S5'(EU419870))				
S6	mdopS6(S6(AB094495), S12(AB105061), S6a)					
S7	mdopS7(S7(U19792), Sd(AB032246), S34(EU310474), S35(EU391605))					
S8	mdopS8(S8(AY744080))					
S9	mdopS9(S9(U19793), Sc(D50836))	msypS9 (S9'(EU419868))	msipS9(S9'(GU727866))			
S10	mdopS10(S10(AB428428), Si(AB052683))					
S11	mdopS11(S11(AB105060), S13, S14(AB094492))					
S12						
S13						
S14						
S15						
S16	mdopS16(S16c(AB126322))					
S17	mdopS17(S17(AB105062), S19(AB094493), 6b)					
S18						
S19						
S20	mdopS20(Sg(AB096138))		mtrsS20(Sg'(AB096138))			
S21	mdopS21(S21(AB094494))			manS21(S48(FJ535239))		
S22	mdopS22(S27b(AF327222), S16b(AB428430))					
S23	mdopS23(S10b(AF239809))	msypS23 (S23'(EU419867))				
S24	mdopS24(S24(AF016920), Sh(AB032247), S42(EU391613))					
S25	mdopS25(S25(AB428431), Sz(AB062100))					
S26	mdopS26(S26(AF016918))					
S27	mdopS27(S27a(AF016919), S16a(AB428429))					
S28	mdopS28(S28(AF201748), Se(AB035273), S36(EU391606))					
S29	mdopS29(S29(AY039720))				msppS29(S2(FJ943266))	
S30		msypS30 (S36'(EU419865))	mtrsS30(Si(AB035928))			
S31	mdopS31(S31(DQ135990))					
S32	mdopS32(S32(DQ135991))					
S33			msipS33 (S33(Ssi5, AB540121), S34(DQ649477))			
S34	mdopS34(S34(Sad5, AB540122))					
S35			msipS35 (S35(DQ868315))			mpprS35(mpS2)
S36						
S37		msypS37 (S37'(EU419864))				

Table 2 Newly arranged *S*-alleles number including two newly identified *S*-alleles for *Malus* spp (Continue)

<i>Malus domestica</i> Pumilae (Section)	<i>M. sylvestris</i> Pumilae	<i>M. sieversii</i> Pumilae	<i>M. angustifolia</i> Chloromeles	<i>M. kansuensis</i> Sorbomalus	<i>M. prunifolia</i> Pumilae
		<i>M. transitoria</i> Sorbomalus	<i>M. mandshurica</i> Gymnomeles	<i>M. spectabilis</i> Pumilae	
S38	msypS38 (S38(EU419863))				
S39	msypS39 (S39(EU419871))				
S40	msypS40 (S40(EU419869))				
S41	msypS41 (S41(EU419872))				
S42	mdopS42(S42(EU427453))				
S43	mdopS43(S43(EU427452))				
S44	msypS44 (S44(EU419862))				
S45	msypS45 (S45(EU419861))				
S46	mdopS46(S46(FJ008672))				
S47	msypS47 (S47(EU419859))				
S48		msipS48 (S48(GU727867))			
S49		msipS49 (S49(FJ535240))			
S50	msypS50 (S50(FJ535241))				
S51				mkasS51(S51(FJ535242))	
S52			mmagS52(S52(FJ535243))	msppS52(S4(FJ943270))	
S53	mdopS53(S53(FJ602074))			msppS53(S3(FJ943268))	
S54	mdopS54(S54(FJ602075), S45(FJ008671))	msypS54 (S46(EU419860))			
S55	mdopS55(Skb(EU443101), S44(FJ008673))				
S56				msppS56(S1(FJ943264))	
S57				msppS57(S5(FJ943272))	mprpS57(mpS1)

The *S-RNases* were published and registered with NCBI: Sa, Sb Sf, Sc: Sassa et al., (1996); S2, S3, S5, S7, S9: Broothaerts et al., (1995); S4, S10b: Van Nerum et al., (2001); Sd: Kitahara et al., (2000); S6, S11, S12, S14, S17, S19, S21: Matsumoto et al., (2003); S8, S-RNase I: Li et al., (2004), Okuno (2000); Si: Kitahara and Matsumoto (2002a); S27a, S27b: Broothaerts (2003); Sg: Matsumoto et al., (2001a); Sh: Kitahara et al., (2000); S24, S26: Verdoodt et al., (1998); Sz: Kitahara and Matsumoto (2002b); Se: Matsumoto and Kitahara (2000); S28: Schneider et al., (2001); S29: Mitiyahu et al. (2005); St: Matsumoto et al., (2000); S31, S32: Kim et al., (2008); mtrsSg': matsumoto et al. (2001); S33: Li et al. (SEP-2005 directly submitted); msipS34-msipS35: Zhang et al. (MAY-2006, JUL-2006 directly submitted); Skb: (SEP-2007 submitted) Bokszezanin et al., (2009); S34, S35-S42: Zhang et al.(NOV-2007, JAN-2008 directly submitted); Ssi5, Sad5: (JAN 2008 submitted) Matsumoto et al. (2010); mdopS42, mdopS43, msipS9", msipS48: Dreesen,R.S.G. et al. (JAN-2008-directly submitted); msypS1', S5', S9', S23', S36-S41, S44-S47: (JAN 2008-submitted) Dreesen et al (2010); mdopS44, S45, S46: (AUG 2008 submitted) Long et al. (2010); mancS48, msipS49, msypS50, mkasS51, mmagS52, mdopS53, mdopS54: Li et al. (DEC-2008 submitted); msppS1, S2, S3, S4, S5: Zhang et al. (APR-2009 Zhang submitted), Heng et al. (2010).

(S36-S47) were also identified from *M. sylvestris* by Dreesen et al. (2010) and used three different nomenclature. Whether those *S-RNases* sequences have unique functions or not yet known, since some of them are rather similar to previously described *S-RNases*. Here, Larsen et al. (2016) followed the same nomenclature of Matsumoto (2013) where the above mentioned alleles were not included. However, they had used

the names as S44_{dom} and S44_{syl} with different functions.

Therefore, we have re-checked the previously assigned *S*-alleles in database, and propose to rearrange the numbers of 57 *S*-allele of apple (Table 2). Therefore, the *mpS1*- and *mpS2-RNase* were designated as *mprpS57* (*Malus prunifolia* Pumilae S57) and *mprpS35* (*Malus prunifolia* Pumilae S35), respectively. If the newly proposed two *S-RNases* are included

in Genbank would be useful to the apple breeder for further improvement of apple using these *S*-alleles for self-incompatibility.

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References

- Bošković R, Tobutt KR (1999) Correlation of stylar ribonuclease isoenzymes with incompatibility alleles in apple. *Euphytica* 107:29–43
- Broothaerts W (2003) New findings in apple *S*-genotype analysis resolve previous confusion and request the re-numbering of some *S*-alleles. *Theor Appl Genet* 106:703–714
- Broothaerts W, Janssens GA, Proost P, Broekaert WF (1995) cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. *Plant Mol Bio* 127:499–511
- Dantas ACDe M, Peixoto ML, Nodari RO, Guerra MP (2005) Germination of pollen and the development of pollen tubes in apple (*Malus* spp.). *Rev Bras Frutic* 27(3):356–359
- De Nettancourt D (1977) Incompatibility in Angiosperms. In: Frankel R et al. (eds) *Monographs on Theor Appl Genet*. Springer-Verlag, New York pp 28–357
- De Nettancourt D (2001) Incompatibility and incongruity in wild and cultivated plants. Springer-Verlag, New York
- Dreesen RSG, Vanholme BTM, Luyten K, Van Wynsberghe L, Fazio G, Roldan-Ruiz I, Keulemans J (2010) Analysis of *Malus S-RNase* gene diversity based on a comparative study of old and modern apple cultivars and European wild apple. *Mol Breed* 26:693–709
- Gu C, Wang L, Korban SS, Han Y (2015) Identification and characterization of *S-RNase* genes and *S*-genotypes in *Prunus* and *Malus* species. *Can J Plant Sci* 95(2):213–225
- Heng W, Wu J, Wu H, Cao Y, Nishio T, Zhang SJ (2011) Recognition specificity of self-incompatibility in *Pyrus* and *Malus*. *Mol Breed* 28:549–557
- Inomata Y, Kudo K, Masuda T, Bessho H, Wada M, Suzuki K (2004) Growth and fruit productivity habits of columnar type apple selections. *Horticultural Research* 3(4): 392–40
- Ishimizu T, Endo T, Yamaguchi-Kabata Y, Nakamura KT, Sakiyama F, Norioka S (1998a) Identification of regions in which positive selection may operate in *S-RNase* of Rosaceae: Implication for *S*-allele-specific recognition sites in *S-RNase*. *FEBS Letters* 440:337–342
- Ishimizu T, Shinkawa T, Sakiyama F, Norioka S (1998b) Primary structural features of rosaceous *S-RNases* associated with gametophytic self-incompatibility. *Plant Mol Biol* 37:931–941
- Janick J, Cummins JN, Brown SK, Hemmat M (1996) Apples. In: Janick J., Moore J.M. (eds): *Fruit Breeding, Tree and Tropical Fruits*. Vol.2. New York, Wiley:1–77
- Janssens GA, Goderis IJ, Broekaert WF, Broothaerts W (1995) A molecular method for *S*-allele identification in apple based on allele-specific PCR. *Theor Appl Genet* 91:691–698
- Kim H, Kakui H, Kotoda N, Hirata Y, Koba T, Sassa H (2009) Determination of partial genomic sequences and development of a CAPS system of the *S-RNase* gene for the identification of 22 *S* haplotypes of apple (*Malus × domestica* Borkh.). *Mol Breed* 23:463–472
- Kim H, Park J, Hirata Y, Nou I (2008) Molecular characterization of two new *S-RNases* (*S*₃₁' and *S*₃₂') in apple (*Malus × domestica* Borkh.). *J Plant Biol* 51:202–208
- Kim HT, Hattori G, Hirata Y, Kim DI, Hwang JH, Shin YU, Nou IS (2006) Determination of self-incompatibility genotypes of Korean apple cultivars based on *S-RNase* PCR. *J Plant Biol* 49:448–454
- Kitahara K, Matsumoto S (2002a) Sequence of the *S*₁₀ cDNA from McIntosh apple and a PCR-digestion identification method. *HortScience* 37:187–190
- Kitahara K, Matsumoto S (2002b) Cloning of the *S*₂₅ cDNA from McIntosh apple and an *S*₂₅-allele identification method. *J Hortic Sci Biotechnol* 77:724–728
- Kitahara K, Soejima J, Komatsu H, Fukui H, Matsumoto S (2000) Complete sequences of the *S*-genes '*Sd*' and '*Sh-RNase*' cDNA in apple. *HortScience* 35:712–715
- Kobel F, Steinegger P, Anliker J (1939) Weitere Untersuchungen über die Befruchtungsverhältnisse der Apfel- und Birnsorten. *Landw Jb Schweiz* 53:160–191
- Komori S, Soejima J, Abe K, Kato H, Kotoda N, Kudo K (1999) Analyses of *S*-allele genotypes of 'McIntosh', 'Kitakami', 'Worcester Pearmain', etc. *J Jpn Soc Hortic Sci* 68(suppl. 2):94 (article in Japanese)
- Komori S, Soejima J, Abe K, Kotoda N, Kato H (2000) Analysis of *S*-allele genotypes and genetic diversity in the apple. *Acta Hortic* 538:83–86
- Korban SS, Skirvin H (1984) Nomenclature of the cultivate apple. *HortScience* 19:177–180
- Larsen B, Orgaard M, Todam-Andersenv TB, Pedersen C (2016) A high-throughput method for genotyping *S-RNase* alleles in apple. *Mol Breeding* 36:24
- Long S, Li M, Han Z, Wang K, Li T (2010) Characterization of three new *S*-alleles and development of an *S*-allele-specific PCR system for rapidly identifying the *S*-genotype in apple cultivars. *Tree Genet Genomes* 6:161–168
- Matityahu A, Stern RA, Schneider D, Goldway M (2005) Molecular identification of a new apple *S-RNase-S29*-cloned from 'Anna', a low-chilling-requirement cultivar. *HortScience* 40:850–851
- Matsumoto S (2013) Database of *S-RNase*. <http://www.agr.nagoya-u.ac.jp/~hort/apple/>. Updated 19 July 2013

- Matsumoto S (2014) Apple pollination biology for stable and novel fruit production: search system for apple cultivar combination showing incompatibility, semicompatibility, and full-compatibility based on the S-RNase allele database. *Int J Agron* 2014:1–9
- Matsumoto S, Furusawa Y (2005) Genomic DNA sequence of *S*_{16c(=16)}-RNase in apple: re-numbering of *S*_{16(=27a)} - and *S*_{22(=27b)} -allele to *S*_{16a} and *S*_{16b}. *Sci Rep Rac Educ Gifu Univ (Nat. Sci.)* 29:7–12
- Matsumoto S, Furusawa Y, Kitahara K, Komori S, Soejima J (2003) Partial genomic sequences of *S*₆ -, *S*₁₂ -, *S*₁₃ -, *S*₁₄ -, *S*₁₇ -, *S*₁₉ -, and *S*₂₁ -RNases of apple and their allele designations. *Plant Biotechnol* 20:323–329
- Matsumoto S, Hayashi S, Kitahara K, Soejima J (2001a) Genomic DNA sequence encoding *Malus × domestica* Borkh. “Akane”, “Delicious” and *Malus transitoria* S-RNases. *DNA Seq* 12:381–383
- Matsumoto S, Hayashi S, Kitahara K, Soejima J (2001b) Genomic DNA sequence encoding *Malus × domestica* Borkh. “Akane”, “Delicious” and *Malus transitoria* S-RNases. *DNA Seq* 12: 381–383
- Matsumoto S, Kitahara K (2000) Discovery of a new self-incompatibility allele in apple. *HortScience* 35:1329–1332
- Matsumoto S, Kitahara K, Komori S, Soejima J (1999a) A new S-allele in apple, ‘Sg’, and its similarity to the ‘Sf’ allele from Fuji. *HortScience* 34:708–710
- Matsumoto S, Komori S, Kitahara K, Imazu S, Soejima J (1999b) S-genotypes of 15 apple cultivars and self-compatibility of ‘Megumi’. *J Jpn Soc Hortic Sci* 68:236–241
- Matsumoto S, Suzuki M, Kitahara K, Soejima J (2000) Possible involvement of a new S-gene ‘St-RNase’ (Accession No. AB035928) in the wild apple possessing high similarity to the ‘S₃’ and ‘S₅-RNase’ in the Japanese pear. *Plant Physiol* 122:620
- Matsumoto S, Yamada K, Shratake K, Okada K, Abe K (2010) Structural and functional analyses of two new S-RNase alleles, Ssi5 and Sad5, in apple. *J Hortic Sci Biotechnol* 85:131–136
- Sassa H, Mase N, Hirano H, Ikehashi H (1994) Identification of self-incompatibility related glycoproteins in styles of apple (*Malus × domestica*). *Theor Appl Genet* 89:201–205
- Sassa H, Nishio T, Kowyama Y, Hirano T, Koba T, Ikehashi H (1996) Self-incompatibility (S) alleles of the Rosaceae encode members of a distinct class of the T2/S ribonuclease superfamily. *Mol Genet Gen* 250:547–557
- Schneider D, Stern RA, Eisikowitch D, Goldway M (2001) Analysis of S-alleles by PCR for determination of compatibility in the Red Delicious apple orchard. *J Hortic Sci Biotechnol* 76:596–600
- Ushijima K, Sassa H, Tao R, Yamane H, Dandekar AM, Gradziel TM, Hirano H (1998) Cloning and characterization of cDNAs encoding S-RNases from almond (*Prunus dulcis*): primary structural features and sequence diversity of the S-RNases in Rosaceae. *Mol Genet Gen* 260:261–268
- van Nerum I, Geerts M, van Haute A, Keulemans J, Broothaerts W (2001) Re-examination of the self-incompatibility genotype of apple cultivars containing putative ‘new’ S alleles. *Theor Appl Genet* 103:584–591
- Verdoordt L, Van Haute A, Goderis JJ, De Witte K, Keulemans J, Broothaerts W (1998) Use of the multi-allelic self-incompatibility gene in apple to assess homozygosity in shoots obtained through haploid induction. *Theor Appl Genet* 96:294–300
- Vieira J, Morales-Hojas R, Santos RAM, Vieira CP (2007) Different positively selected sites at the gametophytic self-incompatibility pistil S-RNase gene in the Solanaceae and Rosaceae (*Prunus*, *Pyrus*, and *Malus*). *J Mol Evol* 65:175–185
- Yamamoto T, Kimura T, Hayashi T, Ban Y (2006) DNA profiling of fresh and processed fruits in pear. *Breed Sci* 56:165–171