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; Verdoort 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 (Sii5), Malus sieversii...
deploy our newly identified two

Total genomic DNA was extracted from the young leaves by
DNA extraction and PCR amplification at -80°C.

NARO Institute of Fruit Tree Science (Morioka, Japan). The No. JP173432) were collected at the Apple Research Station, Plant materials

Materials and Methods

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).

Thus, 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

Results and Discussion

Nucleotide sequences of newly identified S-RNase genes in ‘Marubakaido’

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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 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).

Fig. 2A, B Comparison of the nucleotide and amino acid sequences of the mpS1- and mpS2-RNase with those of the 12 S-RNases of apple.

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

| S5-RNase          | S22-RNase          | Sh-RNase          | S4-RNase          | S21-RNase          | S32-RNase          |
|-------------------|--------------------|-------------------|-------------------|--------------------|--------------------|
| Malus spectabilis | Pyrus sinkiangensis| Pyrus communis    | Malus × domestica | Pyrus × bretschneider | Pyrus korsshineskyl |
| 99.1%             | 93.5%              | 93.5%             | 92.6%             | 92.3%              | 80.7%              |

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, S4 (92.6%) of Malus × domestica, S21 (92.3%) of Pyrus × 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 Pyrus ussuriensis, 94.3% to S9 of Pyrus × korsshineskyl and 85.4% to S26 of Pyrus × 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
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 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 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 ‘Marubakaido’ and S25-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-RNase 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 ‘Bohnapfel’ (S6bS9S16c) and have been re-numbered as S16 (=27a) 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. soulardii were identified by Long et al. (2010), but later of the same year, 12 new S-RNase alleles
| M. domestica | M. sylvestris | M. sieversii | M. angustifolia | M. kansuensis | M. prunifolia |
|-------------|--------------|-------------|----------------|--------------|--------------|
| Pam | Pam | Pam | | | |
| M. dumetorum | | | | | |
| M. williamsii | | | | | |
| M. rothii | | | | | |
| M. pumilum | | | | | |
| M. sieversii | | | | | |
| M. angustifolia | | | | | |
| M. kansuensis | | | | | |
| M. prunifolia | | | | | |

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

| S1 | mdopS1(Sn(D50837)) | msypS1 | (S1(EU419866)) |
| S2 | mdopS2(Sn(U12199), Sn, S3(DQ219464), S37(EU391617), S38(EU391609), S39(EU3916010), S40(EU391611), S41(EU391612)) | | |
| S3 | mdopS3(Sn(U12200), Sb) | | |
| S4 | mdopS4(Sn(AY327223)) | | |
| S5 | mdopS5(Sn(U197991)) | msypS5 | (S5(EU419870)) |
| S6 | mdopS6(Sn(AB094495), S12(A105061), S6a) | | |
| S7 | mdopS7(Sn(U19792), Sn(AB022246), S34(EU310474), S35(EU391605)) | | |
| S8 | mdopS8(Sn(AY744808)) | | |
| S9 | mdopS9(Sn(U19791), Sn(D50836)) | msypS9 | (S9(EU419868)) | msypS9(S9'(EU727866)) |
| S10 | mdopS10(Sn(AB424282), Sn(AB025283)) | | |
| S11 | mdopS11(Sn(A105060), S13, S14(AB094492)) | | |
| S12 | | | |
| S13 | | | |
| S14 | | | |
| S15 | | | |
| S16 | mdopS16(S16c(AB126322)) | | |
| S17 | mdopS17(S17(A105062), S19(A1050493), Sb) | | |
| S18 | | | |
| S19 | | | |
| S20 | mdopS20(Sg(AB096138)) | mtrS20(Sg'(AB096138)) | |
| S21 | mdopS21(S21(A1050494)) | mtrS21(S21(AB035239)) | |
| S22 | mdopS22(S27b(AF327222), S16c(A128430)) | mtrS22(S27b(AB046311), S16c(A128430)) | |
| S23 | mdopS23(S10b(AF239809)) | msypS23 | (S23(EU419867)) | |
| S24 | mdopS24(S24(AF016920), S6b(A1032247), S42(S391613)) | | |
| S25 | mdopS25(S25(AF016921), Sc(AB062100)) | | |
| S26 | mdopS26(S26(AF016918)) | | |
| S27 | mdopS27(S27b(AF016919), S26(A128429)) | | |
| S28 | mdopS28(S28(AF01748), Sc(AB035237), S30(EU391606)) | | |
| S29 | mdopS29(S29(AF309720)) | msypS29 | (S29(EU493266)) | |
| S30 | mdopS30 | msypS30 | (S30(EU419865)) | mtrS30(Sn(AB035282)) | |
| S31 | | | |
| S32 | mdopS32(S32(DQ135991)) | | | |
| S33 | mdopS33 | mtrS33 | (S33(Sn5, AB540121), S34(DQ649477)) | |
| S34 | mdopS34(S34(Sn5, AB540122)) | | | |
| S35 | mdopS35 | mtrS35 | (S35(EU419863)) | msypS35(mtrS35) | |
| S36 | | | |
| S37 | msypS37 | (S37(EU419864)) | | | |

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

| 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       | mSypS47  | (S47(EU419859)) |
| S47       | mSypS48  | (S48(JT728673)) |
| S48       | mSypS49  | (S49(FJ535240)) |
| S49       | mSypS50  | (S50(FJ535241)) |
| S50       | mKasS51  | (S51(FJ535242)) |
| S51       | mmagS52(S52(FJ943270)) |
| S52       | mSypS53  | (S53(FJ943268)) |
| S53       | mdopS54(S54(FJ3008671)) |
| S54       | mSypS55  | (S55(FJ943264)) |
| S55       | mSypS56(S56(FJ943272)) |
| S56       | mSypS57(S57(FJ943272)) |

The S-RNases were published and registered with NCBI: Sa, Sb Sf, Sg: 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., (2003a); 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); mtrSg*: 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) Bokszczanin et al., (2009); S34, S35-S42: Zhang et al.(NOV-2007, JAN-2008 directly submitted); S45, S46: (JAN 2008 submitted) Dreesen et al. (2010); mSipS34, mSipS35, mSipS48: Dreesen, R.S.G. et al. (JAN-2008 directly submitted); mSipS31, S5', S9', S23', S36-S41, S44-S47: (JAN 2008-submitted) Dreesen et al. (2010); mSipS44, S45, S46: (AUG 2008 submitted) Long et al. (2010); mSipS44, mSipS49, mSipS50, mKasS51, mmagS52, mdopS53, mdopS54: Li et al. (DEC-2008 submitted); mSipS1, 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 S44dum, and S44dyl 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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