Reconsideration of S-genotype Assignments, and Discovery of a New Allele Based on S-RNase PCR-RFLPs in Japanese Pear Cultivars

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The S-genotype assignments in the Japanese pear cultivars, 'Akaho', 'Tanzawa', 'Kimizukawase', 'Choju', 'Ichiharawase' and 'Meigetsu' previously determined by pollination tests have raised some doubt recently. These cultivars were analyzed by S-RNase based PCR-RFLP, and their S-genotype assignments reconsidered. The assignment based on the PCR-RFLP system was in agreement with that determined previously by pollination tests in six cultivars, 'Chojuro', 'Doitsu', 'Kikusui', 'Kosui', 'Taihaku' and 'Yakumo', confirming the applicability of the system. 'Akaho', 'Tanzawa', 'Kimizukawase' and 'Choju' were analyzed and assigned as S_3S_5 , S_4S_5 , S_1S_5 and S_7S_5 , respectively. Two of these cultivars, 'Akaho' and 'Tanzawa', were also confirmed by our pollination tests. In three cultivars, 'Ichiharawase', 'Meigetsu' and 'Heiwa' ('Nijisseiki' × 'Ichiharawase'), a new S-RNase fragment with a size and digestion pattern distinct from those of S_1 to S_7 -RNases of Japanese pear was amplified. The deduced amino acid sequence in the hypervariable region of this S-RNase was quite different from those of S_1 to S_7 -RNases. We, therefore, assigned this S-RNase as S_8 -RNase, and identified the S-genotypes of 'Ichiharawase', 'Meigetsu' and 'Heiwa' as S_7S_8 , S_7S_8 and S_4S_8 , respectively.

Key Words: Japanese pear, S-genotype, PCR-RFLP, S_8 -RNase.

Introduction

Analysis of S-genotype in the self-incompatibility of Japanese pear has been used as an aid for both the breeding and management of pollination in orchards. Dealing with the study on the genetic principal of paternal incompatibility

(Kikuchi 1929), Terami *et al.* (1946) demonstrated that seven alleles (S_1 to S_7) were involved in 22 cultivars. Since then, these S-genotype assignments have been used as cross indicators for identifying the genotype of other cultivars.

To date, the S-genotype has been described for almost 40 Japanese pear cultivars through numerous sets of crossings carried out both for pollination tests and breeding (Terami *et al.* 1946, Ogaki 1958, Machida *et al.* 1982, Hiratsuka *et al.* 1998). Among these cultivars, however, some suggested S-genotypes have remained questionable. Two distinct genotypes of S_7S_2 and S_3S_5 have been reported in 'Akaho' (Terami *et al.* 1946, Hiratsuka *et al.* 1995) and of S_3S_5 and S_3S_4 in 'Tanzawa' (Terami *et al.* 1946, Ogaki 1958). 'Kimizukawase' and 'Choju' were both determined as S_2S_5 by pollination tests (Ogaki 1958, Yasunobu *et al.* 1977), but the absence of the S_2 -allele in 'Choju' was proved by its cross-compatibility with pollen from an S_2 homozygote line (Terai *et al.* 1999). Based on the analysis of stylar proteins, Hiratsuka *et al.* (1998) found that 'Choju' was S_7S_5 , and presumed that its pollen parent 'Kimizukawase' has an identical genotype. However, at the same time, their crossing experiments showed that 'Choju' is compatible with 'Ichiharawase' and 'Meigetsu', which were both assigned the S_7S_5 by Terami *et al.* (1946). Accurate determination of the S-genotype is required to resolve such extant confusion.

In Japanese pear, S-allele-associated stylar glycoproteins with RNase activity (termed S-RNases) have been identified by protein analysis (Sassa *et al.* 1992). Five alleles (S_1 , S_2 , S_3 , S_4 and S_5) have been distinguished by isoelectric focusing (IEF) and seven alleles (S_1 to S_7) by two dimensional gel electrophoresis (2D-PAGE) (Hiratsuka *et al.* 1995, Sassa *et al.* 1993, Ishimizu *et al.* 1996). The cDNAs encoding the S_1 to S_7 -RNases have been isolated and sequenced from four cultivars, 'Imamuraaki' (S_1S_6), 'Nijisseiki' (S_2S_4), 'Hosui' (S_3S_5) and 'Okusankichi' (S_5S_7) (Norioka *et al.* 1996, Ishimizu *et al.* 1998). One hypervariable (HV) region was designated based on the diversity of their deduced amino acid sequences (Ishimizu *et al.* 1998). The introns inserted in the HV regions have also been sequenced from genomic DNA (Ishimizu *et al.* unpublished results).

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Based on the seven *S-RNase* nucleotide sequences, we designed primers from their conserved regions and selected allele-specific restriction endonucleases to establish a PCR-RFLP system for identifying the *S*-genotype in Japanese pear cultivars (Ishimizu *et al.* 1999). However, Ishimizu *et al.* (1999) examined only a few cultivars whose *S*-genotype had been previously determined, 'Imamuraaki', 'Nijisseiki', 'Hosui' and 'Okusankichi'. Therefore, to adopt the PCR-RFLP system for identifying or reconsidering *S*-genotype in Japanese pear, it should be evidenced that the cleaved sites of the endonucleases are conserved on their respective *S*-alleles by generating data that demonstrate the coincidence of *S*-genotype assignment both by pollination tests and PCR-RFLP.

In this study, to reconfirm the applicability of the PCR-RFLP system, we employed six Japanese pear cultivars whose *S*-genotypes had previously been determined by pollination tests. We also analyzed six cultivars, 'Akaho', 'Tanzawa', 'Kimizukawase', 'Choju', 'Ichiharawase' and 'Meigetsu', and reconsidered their *S*-genotype assignments based on the results of the PCR-RFLP analysis and the nucleotide sequencing of their amplified *S-RNase* fragments.

Materials and Methods

Plant material and isolation of genomic DNA

The plant material consisted of 17 cultivars of Japanese pear. Four cultivars (designated as the seven allele set cultivars), 'Imamuraaki' (S_1S_6), 'Nijisseiki' (S_2S_4), 'Hosui' (S_3S_5) and 'Okusankichi' (S_5S_7), were used for the fundamental system. Twelve cultivars with previously reported *S*-genotype assignments and 'Heiwa' were analyzed. Young leaves were collected in April 1998 at the National Institute of Fruit Tree Science (NIFTS), Ministry of Agriculture, Forestry and Fisheries of Japan in Tsukuba, Ibaraki. The leaves were frozen in liquid nitrogen and then stored at -80°C . Genomic DNA was extracted from 0.1 g–0.2 g of leaves by a CTAB method (Doyle and Doyle 1987) using 2X CTAB buffer supplemented with 1% polyvinylpyrrolidone and 0.2% 2-mercaptoethanol.

Amplification of *S-RNase* fragments and restriction enzyme digestion

S-RNase fragments were amplified and digested with *S*-allele specific restriction endonuclease by a modification of the original PCR-RFLP system (Ishimizu *et al.* 1999). First, *S-RNase* fragments were amplified using two *S-RNase* specific primers, 'FTQQYQ' (5'-TTTACGCAGCAATATCAG-3') and 'anti-IIWPNV' (5'-AC(A/G)TTCGGCCAAATAATT-3'), whose nucleotide sequences are conserved among the seven *S-RNases* of Japanese pear. The amplified *S-RNase* fragments were then digested with six *S*-allele specific restriction endonucleases (*SfcI*, *PpuMI*, *NdeI*, *AlwNI*, *HincII*, and *AccII*) at a final concentration of 1–5 U for 2 hr at 37°C . Finally, the amplified and digested *S-RNase* fragments were

electrophoresed on 2% agarose gels and detected under UV fluorescence after ethidium bromide staining.

Sequencing of *S-RNase* fragments

The amplified *S-RNase* fragments and digests were electrophoresed on 2% agarose gels and each separated fragment was extracted using the GENE CLEAN II Kit (Bio 101, Inc.) following the manufacturer's instructions. Nucleotide sequences were determined by the dideoxy-nucleotide chain termination method using an ABI PRISM™ 310 DNA capillary sequencer, and analyzed with DNASIS-Mac software (Hitachi Software Engineering Co.).

Pollination tests

After identifying the *S*-genotype in several Japanese pear cultivars using the PCR-RFLP system, some results were confirmed through pollination tests conducted at the orchards of NIFTS in April 1999. Anthers gathered from tester cultivars, 'Hosui', 'Kosui' and 'Doitsu', were dried to collect the pollen. Thirty flowers of 1–2 days before anthesis (at balloon stage) were emasculated, pollinated with the pollen of tester cultivars, and covered with paper bags to avoid contamination. Seventy to 80 days after the pollination, fruit set and seed number were recorded. The crosses were estimated to be incompatible when fruit set was below 30%.

Results and Discussion

Fundamental system of PCR-RFLP for *S*-genotype discrimination

Recently, the PCR-RFLP system has been established for identifying *S*-alleles in Japanese pear cultivars, which is useful, easy and fast (Ishimizu *et al.* 1999). Here, we amplified *S-RNase* fragments from the seven allele set cultivars using 'FTQQYQ' and 'anti-IIWPNV' primers and electrophoresed them on 2% agarose gels instead of 7% polyacrylamide gels to simplify the handling. Agarose gels separated the 'Nijisseiki' S_2 -*RNase* fragment (bearing a 1 kb intron) from the S_1 , S_3 , S_4 , S_5 , S_6 or S_7 -*RNase* fragment (with ca. 170 bp intron). However, S_1 , S_3 , S_4 , S_5 , S_6 and S_7 -*RNase* fragments were detected with similar lengths (referred to as the common fragments) and not separated from each other on agarose gels as they were on polyacrylamide gels (Fig. 1a).

Nevertheless, because discrimination among *S*-alleles is mainly based on specific digestion patterns and not on their sizes, the failure of fragment separation using agarose gels should not interfere with the results, as shown below. *S-RNase* fragments from the seven allele set cultivars, were cleaved with six *S*-allele specific restriction endonucleases, as follows (Table 1). The S_1 -allele was only digested by *SfcI* in 'Imamuraaki', whereas, the S_2 -allele was not digested by any endonuclease in 'Nijisseiki'. The S_3 -allele was only digested by *PpuMI* in 'Hosui' and the S_4 -allele was only digested by *NdeI* in 'Nijisseiki', meanwhile, the S_5 -allele

Fig. 1. Amplification of *S-RNase* fragments from Japanese pear cultivars. a) *S-RNase* fragments amplified from the seven allele set cultivars. 1: 'Imamuraaki' (S_1S_6), 2: 'Nijisseiki' (S_2S_4), 3: 'Hosui' (S_3S_5) and 4: 'Okusankichi' (S_5S_7). b) *S-RNase* fragments amplified from 13 Japanese pear cultivars. 1: 'Chojuro', 2: 'Doitsu', 3: 'Kikusui', 4: 'Kosui', 5: 'Taihaku', 6: 'Yakumo', 7: 'Akaho', 8: 'Tanzawa', 9: 'Kimizukawase', 10: 'Choku', 11: 'Ichiharawase', 12: 'Meigetsu' and 13: 'Heiwa'. In both a) and b): black arrowhead shows the common fragments (CF), arrow the new *S-RNase* fragment, and white arrowhead the *S2-RNase* fragment. DNA sizes are indicated on the left. Fragments were electrophoresed on 2% agarose gel.

was digested both by *Ppu*MI and *Alw*NI in 'Hosui' and 'Okusankichi', and the S_6 -allele was digested both by *Hinc*II and *Acc*II in 'Imamuraaki', finally, the S_7 -allele was only digested by *Acc*II in 'Okusankichi'. The results confirmed the *S*-genotype assignments in all seven allele set cultivars.

Using this modified PCR-RFLP system, we analyzed 13 Japanese pear cultivars and compared their *S*-genotype assignments made by PCR-RFLP analysis with those made by pollination tests.

Applicability of PCR-RFLP for Japanese pear cultivars

Ishimizu *et al.* (1999) examined nine cultivars with previously undetermined *S*-genotype, but they did not utilize cultivars with reported *S*-genotype assignments. In order to use the PCR-RFLP system for identifying or reconsidering the *S*-genotype in Japanese pear, we need to accumulate data that demonstrate the coincidence of *S*-genotypes determined both by pollination tests and PCR-RFLP. We examined six cultivars, 'Chojuro' (S_2S_3), 'Doitsu' (S_1S_2), 'Kikusui' (S_2S_4), 'Kosui' (S_4S_5), 'Taihaku' (S_4S_5) and 'Yakumo' (S_1S_4). These cultivars had *S*-genotype assignments clearly identified by pollination tests (Terami *et al.* 1946, Machida 1966). Moreover, they are currently used in pollination tests as tester cultivars, as well as parental sources for breeding programs (Kajiura and Sato 1990).

Among these cultivars the *S2-RNase* fragment and the common fragments were amplified from 'Chojuro', 'Doitsu' and 'Kikusui'. Solely common fragments were amplified from 'Kosui', 'Taihaku' and 'Yakumo' (Fig. 1b). The common fragments of 'Chojuro', 'Doitsu' and 'Kikusui' were digested by *Ppu*MI (S_3 , S_5), *Sfc*I (S_1) and *Nde*I (S_4), respectively; and, they were assigned the *S*-genotypes of S_2S_3 , S_1S_2 , and S_2S_4 , respectively. Each common fragment in 'Kosui', 'Taihaku' and 'Yakumo' was digested by *Nde*I (S_4) in the three cultivars; by both *Ppu*MI (S_3 , S_5) and *Alw*NI (S_5) in 'Kosui' and 'Taihaku'; and by *Sfc*I (S_1) only in 'Yakumo'. Considering these digestion patterns, the *S*-genotypes of S_4S_5 ,

S_4S_5 and S_1S_4 were assigned to 'Kosui', 'Taihaku', and 'Yakumo', respectively (Table 1).

We showed that in six cultivars, the *S*-genotype assignment by PCR-RFLP coincided with that by pollination tests. This demonstrates that allele-specific restriction endonuclease sites are completely conserved in other cultivars and that PCR-RFLP analysis is a highly reliable method for identifying the *S*-genotype in Japanese pear.

Reconsideration of *S*-genotype assignment in 'Akaho', 'Tanzawa', 'Kimizukawase' and 'Choku'

'Akaho' identified as S_3S_5

'Akaho' was designated S_1S_2 by Terami *et al.* (1946). However, Machida *et al.* (1982) indicated that its *S*-genotype was not clear, because they analyzed two strains of 'Akaho' from Kanagawa and Gumma prefectures which were reciprocally cross-incompatible but compatible with 'Doitsu' (S_1S_2). We re-examined 'Akaho', using both PCR-RFLP and pollination tests. Only common fragments were amplified from 'Akaho' by PCR (Fig. 1b). After the common fragments were cleaved by *Ppu*MI (S_3 , S_5) and by *Alw*NI (S_5) (Fig. 2a), the *S-RNase* fragment not digested by *Alw*NI was sequenced and confirmed to be the S_3 -*RNase*. Thus, the *S*-genotype of S_3S_5 was assigned to 'Akaho' (Table 1). We performed pollination tests in which 'Akaho' was incompatible (0% fruit set) with the pollen of 'Hosui' (S_3S_5), but it was compatible (66.7% fruit set) with the pollen of 'Doitsu' (S_1S_2) (Table 2), thus confirming its assigned *S*-genotype as S_3S_5 . Hiratsuka *et al.* (1995) also suggested that 'Akaho' would be S_3S_5 , based on the analysis of stylar proteins. Considering 'Yakumo' (S_1S_4), which was produced by the cross between 'Nijisseiki' (S_2S_4) and 'Akaho' (Kikuchi 1927), 'Akaho' should have the S_1 -allele, which was not identified in our analysis. It seems that 'Akaho' used in the initial breeding by Kikuchi (1929) is different from that conserved in NIFTS at present.

Table 1. Analysis of the *S*-genotypes of 17 Japanese pear cultivars by PCR-RFLP

Cultivar	<i>S</i> ₂	<i>S</i> ₈	<i>SfcI</i>	<i>PpuMI</i>	<i>NdeI</i>	<i>AlwNI</i>	<i>HincII</i>	<i>AccII</i>	<i>S</i> -genotype	
			<i>S</i> ₁ , <i>S</i> ₈	<i>S</i> ₃ , <i>S</i> ₅	<i>S</i> ₄	<i>S</i> ₅	<i>S</i> ₆	<i>S</i> ₆ , <i>S</i> ₇ , <i>S</i> ₈	PCR-RFLP	Pollination
Seven alleles set:										
Imamuraaki	—	—	+	—	—	—	+	+	<i>S</i> ₁ <i>S</i> ₆	<i>S</i> ₁ <i>S</i> ₆
Nijisseiki	o	—	—	—	+	—	—	—	<i>S</i> ₂ <i>S</i> ₄	<i>S</i> ₂ <i>S</i> ₄
Hosui	—	—	—	++	—	+	—	—	<i>S</i> ₃ <i>S</i> ₅	<i>S</i> ₃ <i>S</i> ₅ ⁽¹⁾
Okusankichi	—	—	—	+	—	+	—	+	<i>S</i> ₅ <i>S</i> ₇	<i>S</i> ₅ <i>S</i> ₇
Test cultivars:										
Chojuro	o	—	—	+	—	—	—	—	<i>S</i> ₂ <i>S</i> ₃	<i>S</i> ₂ <i>S</i> ₃
Doitsu	o	—	+	—	—	—	—	—	<i>S</i> ₁ <i>S</i> ₂	<i>S</i> ₁ <i>S</i> ₂
Kikusui	o	—	—	—	+	—	—	—	<i>S</i> ₂ <i>S</i> ₄	<i>S</i> ₂ <i>S</i> ₄
Kosui	—	—	—	+	+	+	—	—	<i>S</i> ₄ <i>S</i> ₅	<i>S</i> ₄ <i>S</i> ₅
Taihaku	—	—	—	+	+	+	—	—	<i>S</i> ₄ <i>S</i> ₅	<i>S</i> ₄ <i>S</i> ₅
Yakumo	—	—	+	—	+	—	—	—	<i>S</i> ₁ <i>S</i> ₄	<i>S</i> ₁ <i>S</i> ₄
Akaho	—	—	—	++	—	+	—	—	<i>S</i> ₃ <i>S</i> ₅	<i>S</i> ₁ <i>S</i> ₂
Tanzawa	—	—	—	+	+	+	—	—	<i>S</i> ₄ <i>S</i> ₅	<i>S</i> ₃ <i>S</i> ₅
Kimizukawase	—	—	+	+	—	+	—	—	<i>S</i> ₁ <i>S</i> ₅	<i>S</i> ₂ <i>S</i> ₅
Choju	—	—	+	+	—	+	—	—	<i>S</i> ₁ <i>S</i> ₅	<i>S</i> ₂ <i>S</i> ₅
Ichiharawase	—	o	++	—	—	—	—	+	<i>S</i> ₁ <i>S</i> ₈	<i>S</i> ₁ <i>S</i> ₅
Meigetsu	—	o	++	—	—	—	—	+	<i>S</i> ₁ <i>S</i> ₈	<i>S</i> ₁ <i>S</i> ₅
Heiwa	—	o	+	—	+	—	—	+	<i>S</i> ₄ <i>S</i> ₈	Not Determined

o: *S*₂-*RNase* or *S*₈-*RNase* fragment amplified by PCR

++: Two *S*-*RNase* fragments were digested with restriction endonucleases

+: One of two *S*-*RNase* fragments was digested with restriction endonucleases

—: *S*-*RNase* fragments were not digested with restriction endonucleases

¹⁾ The *S*-genotype of 'Hosui' was determined by 2D-PAGE analysis

Table 2. Determination of *S*-genotypes of 'Akaho' and 'Tanzawa' by cross pollination with tester cultivars

Cultivars	PCR-RFLP genotype	Tester Cultivars	No. of Flowers	No. of Fruits	Fruit set (%)	No. of Seeds per fruit	Result ¹⁾
Akaho	<i>S</i> ₃ <i>S</i> ₅	× Doitsu (<i>S</i> ₁ <i>S</i> ₂)	30	20	66.7	4.9	Compat.
		× Hosui (<i>S</i> ₃ <i>S</i> ₅)	30	0	0.0	0.0	Incomp.
Tanzawa	<i>S</i> ₄ <i>S</i> ₅	× Hosui (<i>S</i> ₃ <i>S</i> ₅)	30	21	70.0	5.0	Compat.
		× Kosui (<i>S</i> ₄ <i>S</i> ₅)	30	1	3.3	4.0	Incomp.

¹⁾ The crosses were considered incompatible when fruit set was below 30%.

'Tanzawa' identified as *S*₄*S*₅

Two *S*-genotype assignments have been reported for 'Tanzawa' based on pollination tests, *S*₃*S*₅ (Terami *et al.* 1946) and *S*₃*S*₄ (Ogaki 1958). We re-examined the *S*-genotype of 'Tanzawa' both by PCR-RFLP and pollination tests. Only the common fragments were amplified from 'Tanzawa' by PCR (Fig. 1b). The common fragments were cleaved by *PpuMI* (*S*₃, *S*₅), *NdeI* (*S*₄) and *AlwNI* (*S*₅) (Fig. 2b). The *S*-*RNase* fragments undigested by *NdeI* and *AlwNI* were sequenced and confirmed to be *S*₅- and *S*₄-*RNase*, respectively. Thus, the *S*-genotype of *S*₄*S*₅ was assigned to 'Tanzawa' (Table 1). We also showed that 'Tanzawa' was incompatible (3.3% fruit set) with the pollen from 'Kosui' (*S*₄*S*₅) but was compatible (70% fruit set) with the pollen from 'Hosui' (*S*₃*S*₅) (Table 2), confirming its assigned *S*-genotype. However, since this cultivar is considered as a hybrid of 'Chojuro' (*S*₂*S*₃) × 'Nijisseiki' (*S*₂*S*₄) (Kikuchi 1927), it is not expected to have the *S*₅-allele which is absent in its parents. Thus, the genealogical background of this cultivar

needs to be reconsidered.

'Kimizukawase' and 'Choju' identified as *S*₁*S*₅

'Kimizukawase' is considered as the progeny of 'Shinkozo' × 'Doitsu' (*S*₁*S*₂), and 'Choju' as that of 'Asahi' (*S*₄*S*₅) × 'Kimizukawase' (Kajiura and Sato, 1990). 'Kimizukawase' and 'Choju' were both determined to have the *S*-genotype of *S*₂*S*₅ by pollination tests (Ogaki 1958, Yasunobu *et al.* 1977). However, cross-pollination with pollen from an *S*₂ homozygote line revealed the absence of the *S*₂-allele in 'Choju' (Terai *et al.* 1999).

We re-examined 'Kimizukawase' and 'Choju' by PCR-RFLP. Only common fragments were amplified in both cultivars (Fig. 1b). The common fragments were cleaved by *SfcI* (*S*₁), *PpuMI* (*S*₃, *S*₅) and *AlwNI* (*S*₅) (Fig. 2c). The *S*-*RNase* fragments undigested by *SfcI* and *AlwNI* were sequenced and confirmed to be *S*₅- and *S*₁-*RNase*, respectively. Thus, the *S*-genotype of *S*₁*S*₅ was assigned to both 'Kimizukawase' and 'Choju' (Table 1). This assign-

Fig. 2. Analyses of the digestion pattern of *S*-RNase fragments in four Japanese pear cultivars. a) 'Akaho', b) 'Tanzawa', c) 'Choju', d) 'Ichiharawase'. The lanes in each gel show, from left to right, 1: amplified *S*-RNase fragments, 2: *SfcI* (S_1 , S_8 -specific), 3: *PvuMI* (S_3 , S_5 -specific), 4: *NdeI* (S_7 -specific), 5: *AlwNI* (S_5 -specific), 6: *HincII* (S_6 -specific) and 7: *AccII* (S_6 , S_7 , S_8 -specific). CF: common fragments, DF: digested fragments. DNA sizes are indicated on the left. *S*-RNase fragments and digested fragments were electrophoresed on 2% agarose gels.

ment is consistent with those for three progenies, 'Shinsui' (S_4S_5) ['Kikusui' (S_2S_4) $\times$ 'Kimizukawase'], 'Hayatama' (S_1S_2) ['Gion' (S_2S_4) $\times$ 'Kimizukawase'], and 'Aikansui' (S_4S_5) ['Choju' $\times$ 'Tama' (S_4S_5)] (Kajiura and Sato 1990, Hiratsuka *et al.* 1998).

A new *S*-RNase designated as the S_8 -allele

Using PCR-RFLP analysis, we re-examined 'Ichiharawase' and 'Meigetsu', which were both identified to have the *S*-genotype of S_1S_5 (Terami *et al.* 1946). In 'Ichiharawase' and 'Meigetsu', the common fragment and a new *S*-RNase fragment with a slightly larger size were detected

by PCR (Fig. 1b). This new *S*-RNase fragment was also detected in 'Heiwa' ('Nijisseiki' $\times$ 'Ichiharawase') (Kajiura and Sato 1990), but not in the other 14 cultivars (Fig. 1a and 1b). The presence of the new *S*-RNase fragment in 'Heiwa', the progeny of 'Ichiharawase', shows that it was inherited in a Mendelian manner like the other *S*-alleles of Japanese pear. The primers used in this PCR-RFLP system (Ishimizu *et al.* 1999) were not designed to amplify the *Non S*-RNase gene that was isolated by Norioka *et al.* (1996). The cultivar specificity and the inheritance of the new *S*-RNase fragment, as well as the primer specificity support that this new *S*-RNase is not a *Non S*-RNase.

After the new *S*-RNase fragments from 'Ichiharawase', 'Meigetsu' and 'Heiwa' were electrophoresed on 2% agarose gels, they were extracted and then sequenced. The new *S*-RNase fragment from each cultivar were found to have the same nucleotide sequence of 436 bp, including the intron of 234 bp deduced from the presence of plant 5' and 3' splice-site consensus sequences, which is comparable in location to those of Japanese pear *S*-RNases (Fig. 3). This new *S*-RNase fragment was digested by specific restriction endonucleases for both *SfcI* (S_1) and *AccII* (S_6 , S_7), producing 255 bp and 181 bp fragments (*SfcI*), and 251 bp and 185 bp fragments (*AccII*), that confers a unique digestion pattern distinct from those of S_1 to S_7 -RNases (Fig. 2d). The common fragments amplified from 'Ichiharawase', 'Meigetsu' and 'Heiwa' were identified as S_1 -RNase, S_1 -RNase and S_4 -RNase, respectively (Fig. 2d, Table 1).

Comparing the deduced amino acid sequence of the new *S*-RNase fragment with those from S_1 to S_7 -RNases of Japanese pear (Fig. 3), we found that this new *S*-RNase possessed the conserved region with one histidine residue essential for the RNase activity (Kawata *et al.* 1989), and three potential N-glycosylation sites with the consensus sequence Asn-X-Ser/Thr (X is not Pro and Asp).

The HV region is considered to control allelic specificity in self-incompatible reactions (Matton *et al.* 1997). The HV region of the new *S*-RNase is quite different from those of S_1 to S_7 -RNases. These differences in the HV region indicate that 'Ichiharawase', 'Meigetsu' and 'Heiwa' possess the eighth *S*-RNase (termed S_8 -RNase) that corresponds to the S_8 -allele. We identified the S_8 -RNase and assigned the *S*-genotypes of S_1S_8 , S_1S_8 and S_4S_8 to 'Ichiharawase', 'Meigetsu' and 'Heiwa', respectively (Table 1).

Terami *et al.* (1946) described 'Ichiharawase' and 'Meigetsu' to have the *S*-genotype of S_1S_5 based on a reciprocal cross-incompatibility and a paterclinal incompatibility in the progeny of 'Okusankichi' (S_5S_7) $\times$ 'Meigetsu'. In this study, we have assigned S_1S_5 to the cultivar 'Choju', which showed compatibility with 'Ichiharawase' and 'Meigetsu' (Hiratsuka *et al.* 1998), therefore, 'Ichiharawase' and 'Meigetsu' must not have the *S*-genotype of S_1S_5 . The paterclinal incompatibility reported by Terami *et al.* (1946), can also be explained by the S_1S_7 genotype of 'Meigetsu', but, the S_7 -RNase has not been detected so far in our studies, while the S_8 -RNase was detected and sequenced. As a result,

Fig. 3. Alignment of deduced amino acid sequences of the *S*-RNases in Japanese pear between primers 'FTQQYQ' and 'Anti-IIWPNV'. The hypervariable (HV) region is shadowed. Cysteine residues are boxed. One histidine residue essential for RNase activity is boxed in black. Gaps are marked by dashes. The site of the intron is shown above the sequences.

the *S*-genotype of 'Meigetsu' was assigned as *S₇S₈*.

In the primary project of Japanese pear breeding, most of the major cultivars were released from the selections based on 'Nijisseiki' and/or local cultivars preferentially originating in the Southern Kanto district, such as Kanagawa and Chiba prefectures (Kajiura and Sato 1990). Those cultivars have since dominated and shared most of the production throughout Japan. Other local cultivars like 'Ichiharawase' and 'Meigetsu', possibly originated in Kochi and Ishikawa prefectures, respectively (Kajiura and Sato 1990), were also used for breeding in the past. However, their progenies such as 'Heiwa' ('Nijisseiki' × 'Ichiharawase'), 'Kogetsu' and 'Kubonashi' ('Meigetsu' × 'Nijisseiki') or 'Shingetsu' and 'Enoshima' ('Meigetsu' × 'Shinchu') remained in less diffusion. The *S₈*-allele harboring in 'Ichiharawase' and 'Meigetsu' was distributed in restricted areas and did not contribute to major breeding cultivars. Therefore, the *S*-genotype of 'Meigetsu' could not be reconfirmed and was regarded as *S₇S₅* (Terami *et al.* 1946) for a long time.

Based on the results of PCR-RFLP analysis, we reconsidered the *S*-genotype assignments of six Japanese pear cultivars, 'Akaho' (*S₃S₅*), 'Tanzawa' (*S₄S₅*), 'Choju' (*S₇S₅*), 'Kimizukawase' (*S₇S₅*), 'Ichiharawase' (*S₇S₈*) and 'Meigetsu' (*S₇S₈*). The new *S₈*-allele exists in three cultivars, 'Ichiharawase', 'Meigetsu' and 'Heiwa'. This identification is extremely difficult to achieve through cross-pollination tests because cultivars with a new allele will be compatible with tester cultivars for *S₁* to *S₇*-alleles. Since the PCR-RFLP system is not affected by factors related to pollination tests such as weather conditions, parthenocarpy, availability of tester cultivars, etc, it has proved to be a powerful tool in identifying the *S*-genotype and analyzing new alleles.

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