Two Novel Self-compatible S Haplotypes in Peach (Prunus persica)

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Peach (Prunus persica) as a species is self-compatible (SC), although most other Prunus fruit tree species are partially or fully self-incompatible. We previously identified 3 mutated S haplotypes, S1, S2, and S2m, that confer self-compatibility on commercial peach cultivars for fruit production. In this report, we identified 2 novel SC S haplotypes, S3 and S4, among 130 peach cultivars and strains consisting mainly of ornamental cultivars and wild strains. The S3 haplotype was found only in ornamental cultivars, while the S4 haplotype was found mainly in wild strains. S-RNase s in the S3 and S4 haplotypes appeared to have no defects in their primary structures. S-RNase downstream of the S3- and S4-RNases appeared to have no defects in their primary structures. S-RNase s in the S3 and S4 haplotypes were also present in the S locus downstream of the S-RNase s. These SFB sequences were present in a reverse transcriptional orientation as has been reported in most other functional Prunus S haplotypes; however, both SFB3 and SFB4 appeared to be mutated. DNA sequencing of the entire downstream region of SFB4, extending about 12 kbp to the stop codon of S-RNase, revealed the presence of a premature stop codon 975 bp downstream from the SFB3 start codon. No sequence homologous to SFB downstream of the stop codon was found. There was a 4946 bp insertion in the middle of SFB4. The original SFB4 sequence, obtained by removing the inserted sequence, encodes a typical SFB. Based on the 3 previously identified peach S haplotypes, we supposed that the S3 and S4 haplotypes were also SC pollen part mutant (PPM) S haplotypes. Here, we also discuss possible reasons for all peach S haplotypes identified so far having the PPM SC S haplotype.

Key Words: F-box protein, pollen part mutation, self-incompatibility, SFB, S-RNase.

Introduction

Self-incompatibility is a genetically controlled pollen-pistil recognition mechanism that prevents self-fertilization and promotes outcrossing (de Nettancourt, 2001). Most Prunus (family Rosaceae) fruit tree species exhibit a homomorphic gametophytic self-incompatibility (GSI) system in which self/nonself-recognition is controlled by a single multiallelic locus, called the S locus (Tao and Iezzoni, 2010; Yamane and Tao, 2009). A self-incompatibility reaction is triggered when the same S-allele specificity is expressed in both the pollen and pistil. Thus, growth of a pollen tube bearing either of the 2 S-allele specificities carried by the recipient pistil is arrested in the style. During the last 2 decades, the molecules involved in GSI recognition have been identified in several plant species. It is now known that 2 separate genes, the S-ribonuclease gene (S-RNase) and S haplotype-specific F-box protein gene (SFB) at the S locus, control male and female specificities, respectively, in Prunus (Ushijima et al., 2003; Yamane et al., 2003). The term “S haplotype” is used to describe variants of the S locus, whereas the term “S allele” is used to
describe the variant of a given S locus gene.

Mutations in S-RNase that lead to dysfunction of the S-RNase enzyme are known to confer self-compatibility commonly in rosaceous and solanaceous plants that have the S-RNase-based GSI system. In sour cherry (P. cerasus) (Yamane et al., 2001), Japanese plum (P. salicina) (Watari et al., 2007), and almond (P. dulcis) (Hanada et al., 2009), self-compatibility is conferred by a low level of S-RNase transcription that leads to a low level of S-RNase accumulation in the style. A frameshift or substitution mutation in S-RNase that led to the translation of a dysfunctional S-RNase was also reported to confer self-compatibility in sour cherry (Tsukamoto et al., 2008, 2010). Mutations in the pollen S gene, however, resulted in different outcomes depending on the taxon or the family that showed the S-RNase-based GSI. Although mutations that disrupt the pollen S determinant F-box gene in Solanaceae and Plantaginaceae are supposed not to confer self-compatibility, these mutations did result in self-compatibility in Prunus (Sonneveld et al., 2005; Tao and Iezzoni, 2010; Ushijima et al., 2004; Sonneveld et al., 2007). The S1 haplotype is a pollen part mutant (PPM) version of the almond S haplotype, while the S2 haplotype is a PPM version of the Japanese plum S haplotype. The S2m haplotype is a mutant version of the peach S haplotype, in which both S-RNase and SFB are mutated, while only SFB is mutated in the S1 haplotype. Considering that most Japanese commercial peach cultivars for fruit production are descended from ‘Shanhai Suimitsuto’ (Shang Hai Shui Mi Tao), a Chinese cultivar known as ‘Chinese Cling’ (Yamamoto et al., 2003), there should be unidentified novel peach SC S haplotypes in cultivars and wild strains that originated from other regions.

In this study, we identified 2 novel SC S haplotypes, S′ and S″, among 130 peach cultivars and strains consisting mainly of ornamental cultivars and wild strains. The S-RNAses in the S′ and S″ haplotypes appeared to be intact, while the SFBs in both S haplotypes were truncated. As reported previously for the 3 identified peach S haplotypes, the S′ and S″ haplotypes were assumed to be PPM SC S haplotypes. Here, we discuss the possible reasons why all peach S haplotypes identified so far are PPM SC S haplotype.

**Materials and Methods**

**Plant materials**

A total of 130 peach cultivars and strains consisting mainly of ornamental cultivars and wild strains were selected from peach germplasm collections at the University of California at Davis (USA), the NARO Institute of Fruit Tree Science (Japan), the Research Institute for Agriculture Okayama Prefectural Technology Center for Agriculture, Forestry and Fisheries (Japan), and the Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón (Spain). The origin and description of all cultivars analyzed are shown in Table 1. In addition to the 130 cultivars and strains, 2 Japanese fresh fruit cultivars, ‘Shimizuhakuto’ (S′S2m) and ‘Chiyomaru’ (S″S4), grown at the experimental farm of Kyoto University, were used as references for the S haplotypes in this study. Young leaves were collected in the spring of 2005–2007, frozen in liquid nitrogen, lyophilized, and stored at −20°C until used.

**DNA extraction**

Total DNA was isolated from lyophilized young leaves using the CTAB method or the Nucleon Phytopure plant and fugal DNA extraction kit (GE Healthcare, Piscataway, NJ, USA) as described previously (Hanada et al., 2009).

**PCR-based genotyping**

Total isolated DNA was used as a template for PCRs using the Pru-C2 and Pru-C4R primer set as described previously (Tao et al., 1999). This primer set was designed to detect the length polymorphism in the second intron in S-RNase. Because it appeared that PCRs using the Pru-C2/Pru-C4R primer set were unable to amplify S′R-Nase effectively, we occasionally performed S′R-Nase allele-specific PCRs using the S4-RNase F3 and S4-RNase R5 primer set to determine the presence of the S′R-Nase allele when it was present heterozygously with other S-RNase alleles. A primer set for the dCAPS marker, S2Dra-F and S2Dra-R, was used to distinguish between S′R-Nase and S″R-Nase, as described by Tao et al. (2007). The oligonucleotide primer sequences used in this study are listed in Table 2.

**DNA gel blot analysis**

Five micrograms of total DNA was digested using EcoRI or HindIII, run on 0.8% agarose gel, and transferred to a nylon membrane (Biodyne Plus; Pall, Port Washington, NY, USA). Hybridization was performed using a DIG-dUTP-labeled probe (Roche Diagnostics, Basel, Switzerland) obtained by PCR labeling with sweet cherry S′R-Nase cdNA and the Pru-C2/Pru-C4R primer set, and washed under low stringency conditions, as described previously (Tao et al., 1999). Hybridization signals were detected using chemiluminescent substrate CDP-Star (New England Biolabs, Ipswich, MA, USA).
### Table 1. Cultivars and strains used in this study and their \( S \) haplotypes.

| No. | Cultivar or strain | \( S \) haplotype | Planting location | Origin |
|-----|-------------------|-------------------|------------------|--------|
| 1   | Nepal Peach Col. No. 84-102 | \( S^S \) | NIFTS | Nepal |
| 2   | Nepal Peach Col. No. 84-114 | \( S^S \) | NIFTS | Nepal |
| 3   | Nepal Peach Col. No. 84-120 | \( S^S \) | NIFTS | Nepal |
| 4   | Nepal Peach Col. No. 84-125 | \( S^S \) | NIFTS | Nepal |
| 5   | Nepal Peach Col. No. 84-131 | \( S^S \) | NIFTS | Nepal |
| 6   | Nepal Peach Col. No. 84-133 | \( S^S \) | NIFTS | Nepal |
| 7   | Nepal Peach Col. No. 84-137 | \( S^S \) | NIFTS | Nepal |
| 8   | Nepal Peach Col. No. 84-155 | \( S^S \) | NIFTS | Nepal |
| 9   | Nepal Peach Col. No. 84-B-201 | \( S^S \) | NIFTS | Nepal |
| 10  | Nepal Peach Col. No. 84-B-206 | \( S^S \) | NIFTS | Nepal |
| 11  | Nepal Peach Col. No. 85-119-B | \( S^S \) | NIFTS | Nepal |
| 12  | Nepal Peach Col. No. 85-125 | \( S^S \) | NIFTS | Nepal |
| 13  | Nepal Peach Col. No. 85-379 | \( S^S \) | NIFTS | Nepal |
| 14  | Nepal Peach Col. No. 85-4021 | \( S^S \) | NIFTS | Nepal |
| 15  | Nepal Peach Col. No. 85-4022 | \( S^S \) | NIFTS | Nepal |
| 16  | Nepal Peach Col. No. 85-4067 | \( S^S \) | NIFTS | Nepal |
| 17  | Nepal Peach Col. No. 85-4083 | \( S^S \) | NIFTS | Nepal |
| 18  | Nepal Peach Col. No. 85-4087 | \( S^S \) | NIFTS | Nepal |
| 19  | Nepal Peach Col. No. 85-4092 | \( S^S \) | NIFTS | Nepal |
| 20  | Nepal Peach Col. No. 86-B-204 | \( S^S \) | NIFTS | Nepal |
| 21  | Pakistan Prunus Col. No. 95-26 | \( S^S \) | NIFTS | Pakistan |
| 22  | Pakistan Prunus Col. No. 95-27 | \( S^S \) | NIFTS | Pakistan |
| 23  | 1470.9 B | \( S^S \) | UC Davis | Pakistan |
| 24  | 1474.10 B | \( S^S \) | UC Davis | Pakistan |
| 25  | 1475.10 C | \( S^S \) | UC Davis | Pakistan |
| 26  | 1477.10 B | \( S^S \) | UC Davis | Pakistan |
| 27  | Churkoc | \( S^S \) | UC Davis | Pakistan |
| 28  | Hunshu | \( S^S \) | UC Davis | Pakistan |
| 29  | Thulu | \( S^S \) | UC Davis | Pakistan |
| 30  | Hekito (Double colored) | \( S^S \) | Okayama | China (Ornamental Peach) |
| 31  | Okayama Yaseitou Asahikawa-2 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 32  | Okayama Yaseitou Kamogawa-1 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 33  | Nagano Yaseitou-Wase | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 34  | Noto 3 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 35  | Terute Suimitsu | \( S^S \) | NIFTS | Japan (Ornamental Peach) |
| 36  | Nepal Peach Col. No. 84-115 | \( S^S \) | NIFTS | Nepal |
| 37  | Nepal Peach Col. No. 84-119 | \( S^S \) | NIFTS | Nepal |
| 38  | Chalpachu | \( S^S \) | UC Davis | Pakistan |
| 39  | Noto 2 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 40  | Noto 8 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 41  | Jing Hong | \( S^S \) | NIFTS | China |
| 42  | Jing Hong Tao | \( S^S \) | NIFTS | China |
| 43  | Shen Zhou Bai Xue | \( S^S \) | NIFTS | China |
| 44  | Hoko | \( S^S \) | Okayama | China |
| 45  | Tououbo | \( S^S \) | Okayama | China |
| 46  | Hekito (Beni) | \( S^S \) | Okayama | China (Ornamental Peach) |
| 47  | Kimunnu Nakamineyuumei | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 48  | Yaezaki Bantou O.P. No. 1 | \( S^S \) | NIFTS | Japan (Ornamental Peach) |
| 49  | Okayama Yaseitou Asahikawa-1 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 50  | Nepal Peach Col. No. 84-121 | \( S^S \) | NIFTS | Nepal |
| 51  | Okayama Yaseitou Kamogawa-2 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 52  | Nagano Yaseitou-Barsei | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 53  | Akahayazaki | \( S^S \) | NIFTS | Japan (Ornamental Peach) |
| 54  | Akashidare | \( S^S \) | NIFTS | Japan (Ornamental Peach) |
| 55  | Amami Yaseitou-1 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 56  | Amami Yaseitou-2 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 57  | Chichibu 1 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 58  | Chichibu 4 | \( S^S \) | NIFTS | Japan (Wild Peach) |
| 59  | Nepal Peach Col. No. 84-522 | \( S^S \) | NIFTS | Nepal |
| 60  | Nepal Peach Col. No. 86-B-210 | \( S^S \) | NIFTS | Nepal |
| 61  | Nepal Peach Col. No. 86-V-169 | \( S^S \) | NIFTS | Nepal |
| 62  | Nepal Peach Col. No. 87-VIII-67 | \( S^S \) | NIFTS | Nepal |
| 63  | Pakistan Prunus Col. No. 95-25 | \( S^S \) | NIFTS | Pakistan |
| 64  | Golden Glory | \( S^S \) | NIFTS | United States |
| 65  | Golden Prolific | \( S^S \) | NIFTS | United States |
| 66  | Silver Prolific | \( S^S \) | NIFTS | United States |
| 67  | Swatow | \( S^S \) | NIFTS | China (Ornamental Peach) |
| 68  | Juseitou-Aka-Yae | \( S^S \) | NIFTS | Japan (Ornamental Peach) |
| 69  | Juseitou-Pink-Yae | \( S^S \) | NIFTS | Japan (Ornamental Peach) |
Table 1. Continued

| No. | Cultivar or strain                  | S haplotype | Planting location | Origin                        |
|-----|-----------------------------------|-------------|-------------------|-------------------------------|
| 70  | Da Tao                            | S²S²        | NIFTS             | China                         |
| 71  | Kemomo Nagoshijou                 | S²S²        | NIFTS             | Japan (Wild Peach)            |
| 72  | Ku Tao 1                          | S²S²        | NIFTS             | Taiwan                        |
| 73  | Ku Tao 5                          | S²S²        | NIFTS             | Taiwan                        |
| 74  | Kemomno Okinawamishou-2           | S²S²        | NIFTS             | Japan (Wild Peach)            |
| 75  | Noto 6                            | S²S²        | NIFTS             | Japan (Wild Peach)            |
| 76  | Zao Xia Lu                        | S²S²        | NIFTS             | China                         |
| 77  | Khanda                            | S²S²        | UC Davis          | Pakistan                      |
| 78  | Loimari                           | S²S²        | UC Davis          | Pakistan                      |
| 79  | Shintanyou                        | S²S²        | Okayama           | China                         |
| 80  | Juseitou (Hitoce-Shiro)           | S²S²        | Okayama           | Japan (Ornamental Peach)      |
| 81  | Juseitou (Aka-Yae)                | S²S²        | Okayama           | Japan (Ornamental Peach)      |
| 82  | Okinawa 1                         | S²S²        | NIFTS             | Japan (Wild Peach)            |
| 83  | Yaseitou 5                        | S²S²        | Okayama           | Japan (Wild Peach)            |
| 84  | Yaseitou 6                        | S²S²        | Okayama           | Japan (Wild Peach)            |
| 85  | Yaseitou 7                        | S²S²        | Okayama           | Japan (Wild Peach)            |
| 86  | Terute Beni                       | S²S²        | NIFTS             | Japan (Ornamental Peach)      |
| 87  | Terute Shiro                      | S²S²        | NIFTS             | Japan (Ornamental Peach)      |
| 88  | Okayama Yaseitou Tsugawa-3        | S²S²        | NIFTS             | Japan (Wild Peach)            |
| 89  | Zao Hua Lu                        | S²S²        | NIFTS             | China                         |
| 90  | Chun Lei                          | S²S²        | NIFTS             | China                         |
| 91  | Rikaku Suimitsu                   | S²S²        | Okayama           | China                         |
| 92  | Shang Hai Shui Mi Tao             | S²S²        | NIFTS             | China                         |
| 93  | Fukusyu                           | S²S²        | Okayama           | Taiwan                        |
| 94  | Akabana Bantou                    | S²S²        | NIFTS             | Japan (Ornamental Peach)      |
| 95  | Shidare Hekitou                   | S²S²        | Okayama           | China (Ornamental Peach)      |
| 96  | Yaezaki Bantou                    | S²S²        | NIFTS             | Japan (Ornamental Peach)      |
| 97  | Okayama Yaseitou Asahikawa-3      | S²S²        | NIFTS             | Japan (Wild Peach)            |
| 98  | Fei Chang Tao                     | S²S²        | Okayama           | China                         |
| 99  | Okayama Yaseitou Tsugawa-4        | S²S²        | NIFTS             | Japan (Wild Peach)            |
|100  | Okayama Yaseitou Tsugawa-5        | S²S²        | NIFTS             | Japan (Wild Peach)            |
|101  | Kanhito                           | S²S²        | NIFTS             | Japan (Ornamental Peach)      |
|102  | Shen Zhou Shui Mi Tao             | S²S²        | NIFTS             | China                         |
|103  | Keiho                             | S²S²        | Okayama           | China                         |
|104  | Yaseitou 3                        | S²S²        | Okayama           | Japan (Wild Peach)            |
|105  | Yaseitou 4                        | S²S²        | Okayama           | Japan (Wild Peach)            |
|106  | Okayama Yaseitou Tsugawa-1        | S²S²        | NIFTS             | Japan (Wild Peach)            |
|107  | Okayama Yaseitou Tsugawa-2        | S²S²        | NIFTS             | Japan (Wild Peach)            |
|108  | Kikumomo                          | S²S²        | NIFTS             | Japan (Ornamental Peach)      |
|109  | Sagami Shidare                    | S²S²        | NIFTS             | Japan (Ornamental Peach)      |
|110  | Akita Yaseitou                    | S²S²        | NIFTS             | Japan (Wild Peach)            |
|111  | Chichibu 2                        | S²S²        | NIFTS             | Japan (Wild Peach)            |
|112  | Okayama Yaseitou Koegatouge       | S²S²        | NIFTS             | Japan (Wild Peach)            |
|113  | Noto 5                            | S²S²        | NIFTS             | Japan (Wild Peach)            |
|114  | Ohatsumomo                        | S²S²        | NIFTS             | Japan (Wild Peach)            |
|115  | Hiley                             | S²S²        | UC Davis          | Unknown                       |
|116  | 0664. B                           | S²S²        | UC Davis          | Unknown                       |
|117  | Stanwick                          | S²S²        | UC Davis          | Unknown                       |
|118  | Indian Freestone                  | S²S²        | UC Davis          | Unknown                       |
|119  | 1469.5 B                          | S²S²        | UC Davis          | Pakistan                      |
|120  | 1469.7 B                          | S²S²        | UC Davis          | Pakistan                      |
|121  | 1472.10 B                         | S²S²        | UC Davis          | Pakistan                      |
|122  | 1473.1 B                          | S²S²        | UC Davis          | Pakistan                      |
|123  | 1473.10 B                         | S²S²        | UC Davis          | Pakistan                      |
|124  | Lutkoo                            | S²S²        | UC Davis          | Pakistan                      |
|125  | 1485.6 B                          | S²S²        | UC Davis          | Unknown                       |
|126  | Dai-Shirobana                     | S²S²        | Okayama           | Japan (Wild Peach)            |
|127  | Jeronimo Balate                   | S²S²        | CITA              | Spain                         |
|128  | Jeronimo 2251                     | S²S²        | CITA              | Spain                         |
|129  | Zaitani (Anita)                   | S²S²        | CITA              | United States                 |
|130  | Baby Gold 9                       | S²S²        | CITA              | United States                 |

\(^{a}\) Both S-RNase and SFB genotypes were determined in this study.  
\(^{b}\) NIFTS: NARO Institute of Fruit Tree Science, UC Davis: University of California, Davis, Okayama: Okayama Research Institute for Agriculture, CITA: Unidad de Fruticultura, CITA de Aragón.
Table 2. DNA sequences of oligonucleotide primers used in this study.

| Experiment                         | Primer name         | Sequence (5′-3′)                  | Reference         |
|------------------------------------|---------------------|----------------------------------|-------------------|
| S-RNase-based genotyping           | Pru-C2              | CTATGGCAGAATGTAATTCTAAAACC       | Tao et al., 1999  |
|                                    | Pru-C4R             | GAGTGTTGAGTTGACTTTGGAAGCG        | Tao et al., 1999  |
| dCAPS analysis for S' and S''m     | S2Dra-F             | ACAGAAGTTTGAACTGACTATGAA         | Tao et al., 2007  |
|                                    | S2Dra-R             | CAGCTTATGCGATCTATGCTATTTT        | Tao et al., 2007  |
| S'-RNase-specific amplification    | S4-RNase F3         | GAAAGCCGATGGAACAAGCA             | This work         |
|                                    | S4-RNase R5         | AACTGAGTCTCTTCCTTCTG             | This work         |
| Insert detection for SFB'          | Pp_SFB1_V1F         | TCCACCCAAACAGATGTAAGCG           | This work         |
|                                    | Pp_SFB1_R1          | AAACATAGTTCCTCTGTTCCCGG          | This work         |
| Insert detection for SFB' by dCAPS | Pp_SFB2_BsrBI_F    | GTCGGTCTCAATCCGGTTCGCC          | This work         |
|                                    | Pp_SFB2_R3          | CTCTTACCAACACTATACCT            | This work         |
| Mutation detection for SFB'        | Pp_SFB3_F2          | TCCCTCGGTTATATTG                | This work         |
|                                    | Pp_SFB3_R2N         | AATCCGAGCACAATCAG               | This work         |
| Insert detection for SFB'          | Pp_SFB4_F5          | GTTCCAAACGGACCGAC              | This work         |
|                                    | Pp_SFB4_R2          | GGTATAGCTACCACATGTA             | This work         |

and LAS3000-mini (Fuji Film, Tokyo, Japan) for digital images.

Cloning and characterization of the S' and S'' haplotypes
A fosmid library was constructed from the genomic DNA of ‘Shidare Hekitou’ (S'S'') and ‘Jeronimo Balate’ (S'S'') using the CopyControl Fosmid Library Production Kit (Epicentre, Madison, WI, USA) as described previously (Ushijima et al., 2004). The library was screened using the same DIG-dUTP-labeled sweet cherry S'-RNase cDNA probe as that used for the DNA gel blot analysis. Isolated genomic clones that contained the S' and S'' haplotypes were used as templates for the DNA sequencing reaction and PCR analysis to determine the physical distance between S-RNase and SFB as described previously (Hanada et al., 2009). Deduced amino acid sequences were aligned with other Prunus S-RNases and SFBs using the CLUSTALW program version 1.83 provided by GenomeNet (http://www.genome.jp/tools/clustalw/).

Determination of the mutation in SFB
The SFB allele-specific primer sets used to detect a mutation in SFB were designed to check if a certain cultivar or strain had a mutated SFB (Table 2). All PCR reactions contained 1× ExTaq buffer, 0.2 mM each of dNTPs, 0.4 μM of each primer, 50 ng template total DNA, and 0.4 U TaKaRa ExTaq polymerase (TaKaRa Bio, Shiga, Japan) in a 15-μL reaction volume. PCR amplification was performed using a program with initial denaturation at 94°C for 1 min, 35 cycles of 94°C for 1 min, 56°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR-amplified fragments from SFB', SFB'', and SFB''' were separated directly in 1% agarose gel electrophoresis and visualized with ethidium bromide under UV light. For SFB'', 5 μL of the PCR products were digested with 10 U of BsrBI in a 20-μL reaction volume. Digested SFB'' fragments were separated in 3% agarose gel electrophoresis and visualized with ethidium bromide under UV light.

Results
S-RNase genotyping
The PCRs using the Pru-C2/Pru-C4R primer set to amplify the S-RNases of 130 peach cultivars and strains yielded bands with sizes that were different from the expected sizes from S'- and S''-RNases. As shown in Figure 1, we detected novel fragments of about 600 bp and 1600 bp that were different in size from the bands for the S', S'', and S'''-RNases, which were amplified from several cultivars and strains including ‘Shidare Hekitou’ and ‘Jeronimo Balate’. Because we found that the DNA sequences of the novel PCR bands encoded partial S-RNase sequences, we assigned S' and S'' to the S-RNase alleles revealed by these bands. Because we found only homozygotes for S''-RNase in the PCR analyses, we subjected all 130 cultivars and strains to DNA gel blot analysis using an S-RNase-specific probe (Fig. 2). Several strains and cultivars that had heterozygous genotypes, such as S'3S' and S'S'', were detected; however, no S''S'' genotype was found. Because S'-RNase produced longer PCR fragments than the other peach S-RNase alleles, PCR amplification of the S'-RNase allele seemed to be competitively prohibited when the S'-RNase allele was present along with other S-RNase alleles. Therefore, we occasionally used an S'-RNase-specific primer set to determine the S-RNase genotype of the cultivars and strains. S-RNase genotyping by both DNA gel blot analysis and PCRs corresponded well when the PCR was performed with both Pru-C2/Pru-C4R and the S'-RNase-specific primer sets. Because S'-RNase and S'''-RNase cannot be discriminated by either DNA blot analyses or PCRs with the Pru-C2/Pru-C4R primer set, we used the dCAPS marker to discriminate them. The S-RNase genotypes of all analyzed cultivars determined in this study are shown in Table 1.
Cloning and characterization of S locus genes

Genomic DNA libraries of ‘Shidare Hekitou’ (S2S3) and ‘Jeronimo Balate’ (S4S4) were constructed and screened using an S-RNase gene-specific probe. Confirmation of the presence of SFB and determination of the S-RNase allele was performed by PCR analyses. Full-length DNA sequences for the S3- and S4-RNases were obtained from the genomic clones that were isolated. Both the S3- and S4-RNases seemed to encode an intact S-RNase with no apparent defects. The derived amino acid sequences contained 5 conserved domains, including 2 active sites for RNase catalytic activity, and shared sequence homology with other functional Prunus S-RNase within the range of similarities that was observed between other functional S-RNases (Fig. 3). Unlike S1-, S2-, and S2m-RNases, no S-RNase with high sequence similarity to the S3- or S4-RNases was found in the International Nucleotide Sequence Databases (INSD; http://www.insdc.org/) (Tables 3 and 4). Although SFB sequences were also present in the genomic clones downstream of the S3- and S4-RNases and in reverse transcriptional orientation, as reported in most other functional Prunus S haplotypes, both SFB1 and SFB2 were mutated (Figs. 4 and 5) and appeared to encode truncated dysfunctional SFBs, as was reported previously for peach SFB1 and SFB2 (Fig. 5; Table 5). DNA sequencing of the entire downstream region of SFB extending for about 12 kbp

Fig. 1. PCR based S-RNase genotyping of representative peach cultivars using the Pru-C2/Pru-C4R primer set. The S-RNase genotypes of ‘Shimizuhakuto’ and ‘Chiyomaru’ are known to be S1S2m and S2S2, respectively. The unidentified bands in ‘Shidare Hekitou’ and ‘Jeronimo Balate’ were named S3 and S4, respectively. Lane 1, ‘Shimizuhakuto’ (S1S2m); lane 2, ‘Chiyomaru’ (S2S2); lane 3, ‘Shidare Hekitou’ (S2S3); and lane 4, ‘Jeronimo Balate’ (S4S4).

Fig. 2. S-RNase genotyping by PCR and DNA gel blot analyses. (A) PCR genotyping using the S-RNase-specific Pru-C2/Pru-C4R primer set. (B) S-RNase genotyping by DNA blot analysis with EcoRI digestion. (C) S-RNase genotyping by DNA blot analysis with HindIII digestion. Lanes a, ‘Yaseitou 4’; b, ‘Fei Chang Tao’; c, ‘Nagano Yaseitou-Wase’; d, ‘Nagano Yaseitou-Bansei’; e, ‘Kikumomo’; f, ‘Sagami Shidare’; g, ‘Okayama Yaseitou Asahikawa-2’; h, ‘Okayama Yaseitou Asahikawa-3’; i, ‘Okayama Yaseitou Kamogawa-1’; j, ‘Okayama Yaseitou Kamogawa-2’; k, ‘Okayama Yaseitou Tsugawa-4’; l, ‘Okayama Yaseitou Tsugawa-5’; m, ‘Okayama Yaseitou Tsugawa-6’; n, ‘Dai-Shirobana’, and o, ‘Okayama Yaseitou Koegatouge’.

Fig. 3. Alignment of the deduced amino acid sequences of peach S-RNases. The sequences of the S1-, S2-, and S2m-RNases were reported previously (Tao et al., 2007). Five conserved domains of rosaceous S-RNase (C1, C2, C3, RC4, and C5) are indicated in open boxes. The rosaceous hypervariable region (RHV) is indicated in a gray box. Conserved histidine residues essential for RNase catalytic activity are indicated by open circles, conserved cysteine residues are marked with closed circles, respectively above the alignment. The tyrosine residue in S2m-RNase, which is thought to be mutated from the conserved cysteine residue, is circled. The INSD accession numbers of S1-RNase, S2-RNase, S2m-RNase, S3-RNase, and S4-RNase are AB252415, AB252317, AB597186, AB537563, and AB537565, respectively.

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For peach SFB1, SFB2 (Tao et al., 2007), and SFB4, the putative original sequences that were derived from original P. avium, P. armeniaca, P. dulcis, P. mume, P. armeniaca, P. persica, removing the inserted sequence were used to calculate identities. Pav, P. avium; Par, P. armeniaca; Pd, P. dulcis; Pm, P. mume; Ps, P. persica; Pc, P. cerasus; Psp, P. persica; and Pp, P. persica. The sequences used are as follows; Pav-S1-RNase (AJ298311), Par-S1-RNase (DQ358542), Par-S1-RNase (AY587561), Par-S2-RNase (AY587562), Pd-Sa-RNase (AB026836), Pm-S1-RNase (AB252409), Pm-S1-RNase (AB101438), Pm-S2-RNase (AB252411), Pm-S3-RNase (AB280793), Ps-S1-RNase (EU035975), Ps-S2-RNase (AB252408), Ps-S3-RNase (AB537565), Ps-S4-RNase (AB537566), Pav-SFB2 (AB111519), Pav-SFB13 (DQ385844), Par-SFB1 (AY587562), Par-SFB2 (AB252417), Par-SFB3 (AB537564), and Par-SFB4 (AB537566).

Table 3. Derived amino acid sequence identities (%) of Prunus SFB (upper half) and S-RNases (lower half).

| Species       | Allele | Accession† | Reference                        | Length in the genome (bp) | CDS (bp) | No. amino acid | Note                       |
|---------------|--------|------------|----------------------------------|---------------------------|----------|----------------|----------------------------|
| **P. avium**  | S1     | AB252415   | Tao et al., 2007                 | 1884                      | 693      | 230            |                            |
|               | S2     | AB252417   | Tao et al., 2007                 | 1743                      | 681      | 226            |                            |
|               | S2m    | AB597186   |                                   |                           |          |                | A single amino acid substitution in the C5 region of S2-RNase |
|               | S3     | AB537563   | This work                        | 1197                      | 687      | 228            |                            |
|               | S3     | AB537565   | This work                        | 2150                      | 678      | 225            |                            |
| **P. mume**   | S1     | AB252409   | Tao et al., 2007                 | 1888                      | 693      | 230            | Encoding the same amino acid sequence as P. persica S-RNase |
| **P. salicina** | S1     | AB252411   | Tao et al., 2007                 | 1277                      | 681      | 226            | Encoding the same amino acid sequence as P. persica S-RNase |

† International Nucleotide Sequence Databases (INSD; http://www.insdc.org/) accession number.

For peach SFB, SFB2 (Tao et al., 2007), and SFB13, with 84.4% and 84.3% amino acid identity, respectively (Table 3). Physical distances between S-RNase and SFB in S1 and S2 haplotypes of peach were 12 kb and 4.3 kb, respectively (Fig. 5).

Mutation in SFB

The PCR primer sets that were used to detect mutations in peach SFBs were designed to test if the S haplotypes in all the peach cultivars and strains used in this study were mutated. To detect the presence or absence of the insertion in SFB13, we designed a primer set that amplified the SFB1 region that contained inserted sequences. If the insertion was present, the amplified products would be around 2250 bp.

For peach SFB, SFB2 (Tao et al., 2007), and SFB13, with 84.4% and 84.3% amino acid identity, respectively (Table 3). Physical distances between S-RNase and SFB in S1 and S2 haplotypes of peach were 12 kb and 4.3 kb, respectively (Fig. 5).
Fig. 4. Alignments of the DNA sequences and derived amino acid sequences of peach SFB\(^2\), SFB\(^3\), and \(P.\) avium SFB\(^3\). (A) DNA sequence alignment of \(P.\) avium SFB\(^3\) (PavSFB13), \(P.\) persica SFB\(^3\) (PpSFB3), SFB\(^4\) (PpSFB4) with the 6 bp inserted sequence that contains a stop codon, and SFB\(^4\) reverted by removing the inserted sequence (PpSFB4-4946). The gray box indicates the 6 bp front position of the inserted sequence in peach SFB\(^4\). (B) Amino acid sequence alignment of deduced proteins from \(P.\) avium SFB\(^3\) (PavSFB13), \(P.\) persica SFB\(^3\) (PpSFB3), SFB\(^4\) (PpSFB4), and \(P.\) persica SFB\(^4\) reverted by removing the inserted sequence (PpSFB4-4946). The dotted box indicates the F-box motif. Two of each variable region (V1, V2) and hypervariable region (HV\(a\) and HV\(b\)) are indicated by open and gray boxes, respectively. The INSD accession numbers of \(P.\) avium SFB\(^3\), \(P.\) persica SFB\(^3\), and \(P.\) persica SFB\(^4\) are DQ385844, AB537564, and AB537566, respectively.
be longer than the products from the original intact SFB. We used almond SFB\(^a\), an original intact functional type SFB of SFB\(^b\), as a reference. As shown in Figure 6, SFB\(^b\) from all peach cultivars and strains used in this study yielded longer products than almond SFB\(^b\), indicating that there was no original functional SFB\(^b\) in any of the peach cultivars and strains tested. Because the inserted sequence to SFB\(^b\) was only 5 bp long, it was difficult to distinguish the presence of the insertion by length polymorphism. We therefore developed a dCAPS marker to distinguish the original SFB and the mutated SFB\(^b\) alleles following the strategy used by Ikeda et al. (2004) to develop dCAPS markers for sweet cherry SFB\(^b\). After BsrBI digestion, the PCR product from mutated SFB\(^b\) should be shorter than the product from Japanese plum SFB\(^b\), the original functional type SFB\(^b\) with no insertion. We found that SFB\(^b\) in all the peach cultivars and strains used in this study were mutated SFBs with 5 bp insertions. A reverse primer for the amplification of SFB\(^b\) and a forward primer for SFB\(^b\) were designed from the sequences that were absent in the original functional alleles. Therefore, only mutated SFB alleles were amplified by PCR. All SFB\(^b\) and SFB\(^b\) in the peach cultivars and strains used in this study appeared to be mutated SFBs (Fig. 6).

**Discussion**

This study showed that 2 novel SC PPM S haplotypes were present in peach in addition to the 3 SC PPM S haplotypes, S\(^a\), S\(^b\), and S\(^m\), which were identified previously. Our preliminary survey of the S haplotypes of over 300 diverse peach cultivars and lines indicated that no more novel S haplotypes existed (Hanada and Tao, unpublished data), although some mutated versions of the existing S haplotypes may exist, as seen in the case in S\(^m\) and S\(^b\). The small number of S haplotypes may indicate that peach experienced a population bottleneck and/or positive selection on the mutated SC S haplotypes. Because peach is a domesticated plant, the domestication process may have affected the population bottleneck and/or positive selection on self-compatibility. However, most of the peach-related wild species in the Prunus subgenus Amygdalus, such as P. mira, P. davidiana, and P. kasuensis, are predominantly SC (Tao, Hanada, Akagi and Gradziel, unpublished data), which makes this inference complicated. It is unclear whether the population bottleneck and/or positive selection occurred upon peach

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**Table 5.** Length of peach SFB and their inserted sequence.

| Species | Allele | Accession\(^a\) | Reference | Inserted sequence (bp) | CDS (bp) | No. amino acid | Note |
|---------|--------|-----------------|-----------|------------------------|----------|----------------|------|
| P. persica | SFB\(^b\) | AB252414 | Tao et al., 2007 | 155 | 1098 | 365 | Mutant of P. dulcis SFB\(^b\) with a 155-bp insertion. |
| P. persica | SFB\(^b\) (reverted\(^c\)) | — | — | — | 1128 | 375 | |
| P. persica | SFB\(^b\) | AB252416 | Tao et al., 2007 | 5 | 525 | 174 | Mutant of P. salicina SFB\(^a\) with a 5-bp insertion. |
| P. persica | SFB\(^b\) (reverted) | — | — | — | 1131 | 376 | |
| P. persica | SFB\(^b\) | AB537564 | This work | Unknown\(^w\) | 975 | 324 | Stop codon appeared at the position 975-bp from the start codon |
| P. persica | SFB\(^b\) | AB537566 | This work | 4946 | 399 | 132 | A 4946-bp insertion mutation |
| P. persica | SFB\(^b\) (reverted) | — | — | — | 1131 | 376 | |

\(^a\) International Nucleotide Sequence Databases (INSD; http://www.insdc.org/) accession number.

\(^b\) No. of nucleotide from the start codon to stop codon.

\(^c\) Reverted original allele by removing the inserted sequence.

\(^w\) Neither the downstream sequence or the original stop codon of SFB\(^b\) was found in the 12-kb downstream region from the stop codon of SFB\(^b\) to the stop codon of S-RNase.
Fig. 6. Detection of mutation in the coding regions of peach SFBs by PCR analysis. A specific primer pair for each SFB allele was designed to detect mutation. Open boxes indicate intact coding regions. Start and stop codon positions are indicated by open and closed triangles, respectively. Arrows indicate the positions of the forward (Fw) and reverse (Rev) primers. (A) PCR amplification to detect the insertion in SFB1. Almond SFB1, a wild type of SFB, was used as a control. Lane 1, ‘Jing Hong’; lane 2, ‘Terute Suimitsu’; lane 3, ‘Nagano Yaseitou-Wase’; lane 4, ‘Nagano Yaseitou-Bansei’; lane 5, ‘Noto 2’; lane 6, ‘Noto 3’; lane 7, ‘Noto 8’; lane 8, ‘Yaezaki Bantou O.P. No. 1’; lane 9, ‘Okayama Yaseitou Kamogawa-1’; and lane 10, ‘Okayama Yaseitou Asahikawa-1’.(B) The dCAPS marker to detect inserted sequence in SFB2. P. salicina SFB2, a wild type of SFB2, was used as the control. Amplified fragment from P. persica SFB2 was detected as different sizes after BsrI digestion. Lane 1, ‘Akashidare’; lane 2, ‘Akabana Bantou’; lane 3, ‘Akahayazaki’; lane 4, ‘Amami Yaseitou-1’; lane 5, ‘Amami Yaseitou-2’; lane 6, ‘Da Tao’; lane 7, ‘Okinawa 1’, and lane 8, ‘Kirimu Nakaminewysuimauri’. (C) PCR amplification to detect mutation in SFB3. Lane 1, ‘Kikumomo’; lane 2, ‘Sagami Shidare’; lane 3, ‘Akabana Bantou’; lane 4, ‘Yaezaki Bantou O.P. No. 1’; lane 5, ‘Yaezaki Bantou’, and lane 6, ‘Shidare Hekitoi’. (D) PCR amplification to detect insertion in SFB4. Lane 1, ‘Okayama Yaseitou Asahikawa-1’; lane 2, ‘Okayama Yaseitou Asahikawa-3’; lane 3, ‘Okayama Yaseitou Tsugawa-4’; lane 4, ‘Okayama Yaseitou Tsugawa-5’; lane 5, ‘Okayama Yaseitou Kamogawa-2’; lane 6, ‘Chichibu 2’, lane 7, ‘Noto 5’; lane 8, ‘Okayama Yaseitou Koegatoe’; lane 9, ‘Fei Chang Tao’; lane 10, ‘Ohatsumomo’; lane 11, ‘Akita Yaseitou’; lane 12, ‘Nagano Yaseitou-Bansei’; and lane 13, ‘Dai-Shirobana’.

speciation from its progenitor species or before peach speciation. Population genetic approaches and investigation of the S locus and S haplotype in peach-related Amygdalus species could give important clues to address the question.

In Prunus, dysfunction of either the pistil S determinant S-RNase or the pollen S determinant SFB confers self-compatibility. Thus, if evolutionary constraints or selection could be disregarded, the rate of mutation needed to confer self-compatibility would be equal for both the pistil and pollen parts in Prunus. Although the coding sequence of SFB is 1.5 times longer than that for S-RNase, the S-RNase sequence from the initiation codon to the termination codon is longer than the SFB sequence because of the presence of introns in the S-RNase sequence. Considering that the causal factor of self-compatibility in peach is a mutation in pollen S for all the S haplotypes found, the mutation in pollen S may have been preferentially selected. As we proposed previously (Tao et al., 2007), the mutation in pollen S may have been selected preferentially compared with the pistil part mutants under selection pressure for SC because the pollen genotype determines the self-incompatible phenotype of pollen in the GSI system. Namely, a mutation in SFB that occurs in a single pollen grain could confer self-compatibility to the original pollen grain in which the mutation first occurs. Then the SC phenotype would be transmitted to the second generation, in which the pollen grain would participate in fertilization either after self- or cross-pollination, while a mutation in S-RNase in a single pollen grain would be unable to confer self-compatibility to the pollen and would be only transmittable to the progeny after cross-pollination because mutations in S-RNase would have no effect on the SC/SI phenotype of the pollen grain. We therefore suppose that the mutation in pollen S would be preferentially selected under selection pressure for SC in the GSI system. If our hypothesis is correct, peach has experienced positive selection for SC in its evolutionary path.

On the practical side, this study could give us important indications of how we can breed SC cultivars in Prunus fruit tree species, in which one of the major breeding goals is SC. Current SC breeding in Prunus is exclusively accomplished by cross breeding using existing SC strains as a parent. For example, almost all SC sweet cherry (P. avium) cultivars recently released are offspring of JI2420, which is a SC strain produced by X-ray irradiation breeding (Lewis, 1949; Ushijima et al., 2004). SC ‘NK14’ Japanese apricot (P. mume) is from crosses between self-incompatible ‘Nanko’ and SC ‘Kensaki’, a naturally occurring PPM SC cultivar. However, considering the astronomical number of pollen grains present in a single flower and that a mutation in SFB in a single pollen grain could confer self-compatibility to the pollen grain itself, we should be able to more effectively utilize spontaneous or artificial mutation in SFB for SC breeding, as the SC PPM S‘ haplotype was artificially produced in sweet cherry (Lewis, 1949).

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