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S Locus F-Box Brothers: Multiple and Pollen-Specific F-Box Genes With *S* Haplotype-Specific Polymorphisms in Apple and Japanese Pear

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ABSTRACT

Although recent findings suggest that the F-box genes *SFB/SLF* control pollen-part *S* specificity in the S-RNase-based gametophytic self-incompatibility (GSI) system, how these genes operate in the system is unknown, and functional variation of pollen *S* genes in different species has been reported. Here, we analyzed the *S* locus of two species of Maloideae: apple (*Malus domestica*) and Japanese pear (*Pyrus pyrifolia*). The sequencing of a 317-kb region of the apple S^9 haplotype revealed two similar F-box genes. Homologous sequences were isolated from different haplotypes of apple and Japanese pear, and they were found to be polymorphic genes derived from the *S* locus. Since each *S* haplotype contains two or three related genes, the genes were named *SFBB* for *S* locus *F-b*ox *b*rothers. The *SFBB* genes are specifically expressed in pollen, and variable regions of the *SFBB* genes are under positive selection. In a style-specific mutant *S* haplotype of Japanese pear, the *SFBB* genes are retained. Apart from their multiplicity, *SFBB* genes meet the expected characteristics of pollen *S*. The unique multiplicity of *SFBB* genes as the pollen *S* candidate is discussed in the context of mechanistic variation in the S-RNase-based GSI system.

THE S-RNase-based gametophytic self-incompatibility (GSI) system has been found in the families Solanaceae, Rosaceae, and Scrophulariaceae. Haplotypes of a single *S* locus determine the specificity of self and nonself discrimination; when an *S* haplotype in the haploid pollen matches one of two *S* haplotypes in the pistil, then the pollen is recognized as "self" and rejected by the pistil (de Nettancourt 2001). The *S* haplotype contains two closely linked *S*-specificity genes, pistil *S* and pollen *S*. While pistil *S* has been known to be the *S-RNase* gene, identity of the pollen *S* has long been unknown until recently (Kao and Tsukamoto 2004; McClure and Franklin-Tong 2006). Findings of *SLF/SFB* as the pollen *S* gene suggested that the F-box protein determines the pollen *S* specificity (Entani *et al.* 2003; Sijacic *et al.* 2004; Ushijima *et al.* 2003, 2004). Since the well-documented function of F-box protein is substrate recognition as a component of SCF complex, a kind of E3 ubiquitin ligase, it has been hypothesized that SLF/SFB recognizes nonself S-RNase in compatible pollen tubes and ubiquitinylates it for degradation by the 26S proteasome (Ushijima *et al.* 2003, 2004; Qiao *et al.* 2004; Hua and Kao 2006). However, recent immunolocalization and immunoblot analyses have shown that S-RNase is incorporated into vacuoles inside pollen tubes and that the amount of S-RNase is not significantly different between compatible and incompatible pollinations (Goldraij *et al.* 2006). Consequently, how SLF/SFB and S-RNase interact to trigger the self-incompatibility reaction is still largely unclear.

Although Solanaceae and Prunus species use a similar molecule as the pistil *S* determinant (S-RNase), clear differences have been reported for pollen *S*. First, pollen *S* in Prunus (*SFB*) shows much higher allelic diversity (66–82.5% amino acid identity; Ikeda *et al.* 2004) than pollen *S* (*SLF*) in Solanaceae (88.4–89.4% amino acid identity; Sijacic *et al.* 2004). Second, diploid pollen from the Prunus tetraploid is frequently capable of normal self-incompatibility function (Hauck *et al.* 2006), but heteroallelic pollen from Solanaceae always shows breakdown of self-incompatibility (SI) (competitive interaction) (de Nettancourt 2001). Finally, in Solanaceae, *SLF* is considered to be essential for pollen viability because all the pollen-part mutations were duplications of pollen *S* and no deletion type was recovered even

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AB270792 (genomic sequence of apple S^9 haplotype), AB270793 (*MdSFBB*$^{9\text{-}\alpha}$), AB270794 (*MdSFBB*$^{9\text{-}\beta}$), AB270795 (*MdSFBB*$^{8\text{-}\alpha}$), AB270796 (*MdSFBB*$^{8\text{-}\beta}$), AB270797 (*PpSFBB*$^{1\text{-}\alpha}$), AB270798 (*PpSFBB*$^{1\text{-}\beta}$), AB270799 (*PpSFBB*$^{1\text{-}\gamma}$), AB270800 (*PpSFBB*$^{5\text{-}\alpha}$), AB270801 (*PpSFBB*$^{5\text{-}\beta}$), and AB270802 (*PpSFBB*$^{5\text{-}\gamma}$).

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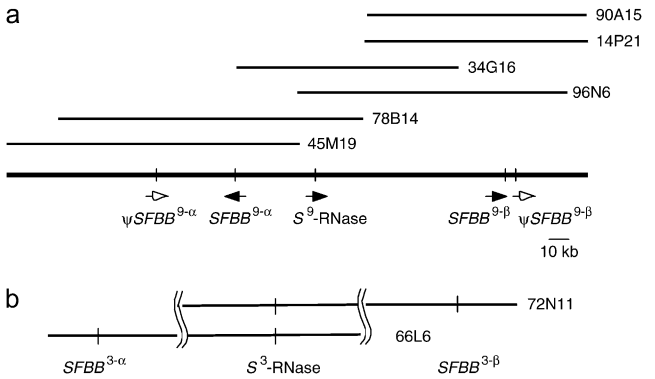

FIGURE 1.—The *S* locus BAC contig of apple. (a) Apple S^9 haplotype. Thin bars are in scale and show BAC clones. Solid arrows denote the transcriptional direction of genes. Open arrows show pseudogenes. (b) BAC clones for the apple S^3 haplotype. Bars are not in scale.

after large-scale screening of X-ray-induced mutants (GOLZ *et al.* 2001). In contrast, deletion of *SFB* results in pollen-part self-compatibility in Prunus (SONNEVELD *et al.* 2005). These differences in pollen *S* may reflect a mechanistic diversity of GSI systems among species.

Rosaceae comprises four subfamilies: Spiraeoideae, Rosoideae, Maloideae, and Amygdaloideae. In species of Maloideae and Amygdaloideae, the GSI mechanism has been studied at a molecular level and S-RNase's have been characterized extensively; however, the pollen *S* gene (*SFB*) has been identified only in Prunus, a species of Amlygdaloideae. The recent finding that Prunus *SFB* barely causes competitive interaction in heteroallelic pollen prompted HAUCK *et al.* (2006) to suggest that pollen *S* in Prunus may be different from pollen *S* in Solanaceae. However, competitive interaction of pollen *S* has been documented in pear (*Pyrus communis*), a species of Maloideae (CRANE and LEWIS 1941; LEWIS and MODLIBOWSKA 1942). Characterization of pollen *S* in Maloideae and comparison of it to its counterparts in Prunus and Solanaceae are likely to shed light on the mechanism and evolution of the S-RNase-based GSI system. However, attempts to isolate pollen *S* in Maloideae through a homology-based approach with Prunus *SFB* sequence information have been unsuccessful (Y. SUZUKI and H. SASSA, unpublished results), most likely because of sequence diversity of pollen *S* between these subfamilies.

Here, we analyzed the apple *S* locus, a species of Maloideae, to identify pollen *S*. A complete sequence of the 317-kb apple S^9 haplotype identified two closely related F-box genes, which we have named *SFBB* (*S* locus *F-b*ox *b*rothers). Two *SFBB* genes also were isolated from apple S^3 haplotype BAC clones, and three *SFBB* genes were isolated in each of the Japanese pear S^4 and S^5 haplotypes. *SFBB* genes in apple and Japanese pear show *S* haplotype-specific sequence polymorphism and pollen-specific gene expression. Analysis showed that S^{4sm}, a mutant Japanese pear haplotype that lacks the S^4-RNase

TABLE 1

Predicted ORFs in the Apple S^9 haplotype

ORF	Homolog, accession no. (*E*-value)
ORF1	*Medicago truncatula* putative retrotransposon, ABE87982 ($2e^{-90}$)
ORF2	*Oryza sativa* retrotransposon, XP_473330 (e^{-59})
ORF3	Unknown
ORF4	Unknown
ORF5	Unknown
ORF6	Unknown
ORF7	*O. sativa* hypothetical protein, ABA95009 ($3e^{-16}$)
ORF8	Unknown
ORF9	Unknown
ORF10	*Solanum demissum* putative retroelement, AAT40504 ($2e^{-43}$)
ORF11	Unknown
ORF12	Unknown
ORF13	*M. truncatula* putative retroelement, ABE91625 ($5e^{-57}$)
ORF14	*M. truncatula* hypothetical protein, ABE93286 ($3e^{-6}$)
ORF15	*O. sativa* putative retroelement, ABA98201 (0)
ORF16	*O. sativa* putative retroelement, ABA98732 ($6e^{-27}$)
ORF17	*O. sativa* putative retroelement, AAV24758 ($4e^{-36}$)
ORF18	Unknown
ORF19	*Arabidopsis thaliana* putative retroelement, AAC33961 ($9e^{-12}$)
ORF20	*Prunus mume* S1-SLFL2, AB092625 ($6e^{-36}$)
ORF21	Unknown
ORF22	*A. thaliana* unknown protein, BAD44360 ($3e^{-8}$)
ORF23	*M. truncatula* putative retroelement, ABE83303 ($3e^{-153}$)
ORF24	Unknown
ORF25	Unknown
ORF26	*Phaseolus vulgaris* putative retroelement, AAR13317 ($8e^{-7}$)
ORF27	*O. sativa* putative retroelement, ABA93344 (e^{-3})
ORF28	Unknown
ORF29	*P. mume* S7-SLFL1, AB092624 ($2e^{-41}$)
ORF30	Unknown
ORF31	Unknown
ORF32	Unknown
ORF33	Unknown
ORF34	*Vitis vinifera* putative retroelement, BAD18986 ($2e^{-23}$)
ORF35	Unknown
ORF36	Unknown
ORF37	Unknown
ORF38	Unknown
ORF39	*Malus x domestica* retrotransposon, AY603367 ($3e^{-122}$)
ORF40	Unknown
ORF41	Unknown
ORF42	*A. thaliana* putative retroelement, AAD15534 ($4e^{-18}$)
ORF43	*M. x domestica* S9-RNase, AY187627 ($4e^{-148}$)
ORF44	*O. sativa* putative retroelement, ABF98943 ($3e^{-68}$)
ORF45	Unknown
ORF46	*O. sativa* putative retroelement, ABA93430 ($3e^{-23}$)
ORF47	Unknown
ORF48	Unknown

(*continued*)

TABLE 1

(Continued)

ORF	Homolog, accession no. (*E*-value)
ORF49	Unknown
ORF50	*O. sativa* putative retroelement, XP_474875 ($4e^{-25}$)
ORF51	Unknown
ORF52	*O. sativa* putative retroelement, AAP52462 ($5e^{-4}$)
ORF53	*O. sativa* putative retroelement, XP_470707 ($2e^{-58}$)
ORF54	*M. truncatula* putative retroelement, ABE94393 (0)
ORF55	Unknown
ORF56	Unknown
ORF57	*M. truncatula* putative retroelement, ABE87633 ($6e^{-8}$)
ORF58	Unknown
ORF59	Unknown
ORF60	*M. truncatula* putative retroelement, ABE90802 ($4e^{-8}$)
ORF61	*P. mume* S1-SLFL1, AB092623 ($2e^{-46}$)
ORF62	Unknown
ORF63	*O. sativa* putative aminotransferase, XP_467987 ($2e^{-7}$)
ORF64	*O. sativa* putative retroelement, NP_919970 ($5e^{-37}$)
ORF65	*O. sativa* putative retroelement, NP_920511 (e^{-42})
ORF66	*O. sativa* putative retroelement, XP_474437 ($8e^{-148}$)
ORF67	*O. sativa* putative retroelement, ABA95102 ($5e^{-50}$)
ORF68	Unknown
ORF69	Unknown
ORF70	Unknown
ORF71	*M. truncatula* putative retroelement, ABE87982 ($4e^{-88}$)

gene and confers pistil-specific self-compatibility, lacks at least 110 kb that contains the S^4-RNase gene but retains three *SFBB*^{4} genes. A sequence analysis also revealed that variable regions of *SFBB* genes are under positive selection. Apart from their multiplicity, the data support the idea that *SFBB* genes are the pollen *S* genes of apple and Japanese pear. The unique multiplicity of *SFBB* genes as the pollen *S* candidate is discussed in the context of functional variation in the S-RNase-based GSI system.

MATERIALS AND METHODS

Plant materials: An apple (*Malus x domestica*) cultivar, Sekaiichi (S^3S^9), and 16 cultivars of Japanese pear [*P. pyrifolia* (syn. *serotina*)]—Hayatama (S^1S^2), Doitsu (S^1S^2), Suisei (S^1S^4), Imamuraaki (S^1S^6), Chojuro (S^2S^3), Kikusui (S^2S^4), Nijisseiki (S^2S^4), Osa-Nijisseiki (S^2S^{4sm}), Chikusui (S^3S^4), Akemizu (S^3S^5), Hosui (S^3S^5), Shinsui (S^4S^5), Kosui (S^4S^5), Hogetsu (S^1S^7), Okusankichi (S^5S^7), and Chukanbohon Nou No.1 ($S^{4sm}S^{4sm}$)—were used. Forty progenies obtained by crossing Chikusui (S^3S^4) and Akemizu (S^4S^5) and 40 plants derived from a cross between Akemizu (S^3S^5) and Shinsui (S^4S^5) were also used.

Construction of BAC and cosmid contigs: A BAC library of the apple cultivar Florina (Vinatzer *et al.* 1998) was obtained from Texas A&M University and screened using the apple S^9-RNase cDNA (S^c-RNase) (Sassa *et al.* 1996) as a probe. Overlapping clones were obtained by screening the library with probes from different positions in the initial BAC clones.

The cosmid library for the cultivar Nijisseiki (S^2S^4; Sassa *et al.* 2002) was screened using the S^4-RNase cDNA (Sassa *et al.* 1997) as a probe. Overlapping clones were then obtained using the method described by Ushijima *et al.* (2001).

Shotgun sequencing: The S^9 haplotype-derived BAC clones (34G16, 45M19, and 90A15) were subjected to shotgun sequencing at Hitachi High-Tech Science Systems (Ibaraki, Japan) (Iwashita *et al.* 2003). For each BAC clone, a fivefold sequence coverage was assembled, and gaps were filled by polymerase chain reaction (PCR) and by direct sequencing of BACs. The assembled sequence was further verified by PCR.

Construction of phylogenetic trees of F-box proteins: Amino acid sequences of F-box proteins were aligned using ClustalX (Thompson *et al.* 1997) and manually optimized. A neighbor-joining tree was constructed using the alignment (Saitou and Nei 1987). Protein distances among pairs of sequences were produced using the PAM Dayhoff matrix (Dayhoff *et al.* 1979) implemented by the PROTDIST program in PHYLIP (Felsenstein 2005). For each distance

TABLE 2

Amino acid sequence identities (%) among the *S* locus-encoded F-box proteins

	MdSFBB9-β	PdSFBa	PdSFBb	PdSFBc	PdSFBd	PdSLFc	PdSLFd	PmS7-SLFL1	PmS7-SLFL2	PmS7-SLFL3	PiSLF2
MdSFBB9-α	87.5	22.6	28.2	25.3	22.5	34.4	34.7	34.7	32.6	34.4	28.9
MdSFBB9-β	—	22.4	23.1	21.3	21.5	36.8	37.0	36.1	33.0	33.2	29.3
PdSFBa		—	69.0	70.1	68.4	25.8	22.8	25.8	40.4	24.9	22.1
PdSFBb			—	75.6	76.4	25.0	23.7	22.9	41.4	44.9	27.8
PdSFBc				—	75.8	25.0	23.7	22.9	41.4	44.9	27.8
PdSFBd					—	21.9	21.5	21.0	36.8	44.9	21.5
PdSLFc						—	95.1	92.5	35.7	47.6	25.4
PdSLFd							—	94.2	35.7	47.3	26.3
PmS7-SLFL1								—	34.6	46.1	25.4
PmS7-SLFL2									—	33.1	30.4
PmS7-SLFL3										—	24.2

Abbreviations for the F-box proteins: Md, apple (*Malus domestica*); Pd, almond (*Prunus dulcis*); Pm, Japanese apricot (*Prunus mume*); Pi, *Petunia inflata*. Sequences of almond, Japanese apricot, and *P. inflata* F-box proteins are from Ushijima *et al.* (2003), Entani *et al.* (2003), and Sijacic *et al.* (2004), respectively.

TABLE 3

Amino acid sequence identities (%) among the SFBBs

	MdSFBB3-β	MdSFBB9-α	MdSFBB9-β	PpSFBB4-α	PpSFBB4-β	PpSFBB4-γ	PpSFBB5-α	PpSFBB5-β	PpSFBB5-γ
MdSFBB3-α	82.2	70.5	69.0	67.3	71.7	63.0	67.3	69.3	62.3
MdSFBB3-β	—	73.7	73.4	71.6	72.5	66.0	71.1	71.8	66.1
MdSFBB9-α		—	87.5	83.9	67.6	70.6	83.7	65.6	70.1
MdSFBB9-β			—	80.6	66.6	68.1	80.9	63.3	67.6
PpSFBB4-α				—	66.9	66.8	96.4	65.3	66.3
PpSFBB4-β					—	60.4	65.9	89.4	59.9
PpSFBB4-γ						—	65.8	58.6	99.0
PpSFBB5-α							—	64.6	65.3
PpSFBB5-β								—	58.4

Pp, Japanese pear (*Pyrus pyrifolia*). See Table 2 legend for other abbreviations.

matrix, a bootstrap analysis was performed by randomly selecting amino acid positions for replacement to produce 1000 replicate protein distance matrices upon which the neighbor joining was performed.

Isolation of nucleic acids: Genomic DNAs were isolated from leaves as described by Doyle and Doyle (1990) and Sassa and Hirano (1998). RNAs were isolated from leaves and floral organs as described by McClure *et al.* (1990).

PCR and RACE: *SFBB*s of the apple S^3 haplotype were amplified from BAC clones 66L6 and 72N11 using primer pairs FMdSL21 (ATGTCCCAGGTGCGTGAAAG) and RMdSL21 (CAATTCACTTGACTGGAACAATAC) and FSMF1 (TACRTGWGAAKAWTTCHYGTG) and RSMF1 (CTCAAGC HTTGTATCATGCATAC), respectively. Flanking sequences of the 66L6-derived gene, $MdSFBB^{\alpha}$, were further amplified using the DNA Walking SpeedUp kit (Seegene, Seoul, Korea) to determine the full-length sequence of the coding region.

Amplification of *SFBB* genes from the Japanese pear cosmid clones and from the Nijisseiki genomic DNA was conducted using primers FjpFB1 (CCAAGTCTCTGATGMGRTTCAAATG) and RjpFB1 (SRGTTAGKWGTTTTGTCCATGAAC), which were designed to amplify all *SFBB* sequences.

Total RNA from the pollen of Kosui was used for 3′RACE, using FMdSL21 as a specific primer. 5′RACE was conducted using specific primers PpSLFLr1 (AGAAGGATACAAGTGGA GGATG) and PpSLFLr2 (AATTGCTGAGGTGTTTGGCC) essentially as described by Ushijima *et al.* (2003). Full-length cDNAs of PpSFBBs were amplified by 3′RACE, using specific primers listed in supplemental Table 1 at http://www.genetics.org/supplemental/.

DNA and RNA blot analysis: Five micrograms of genomic DNAs digested with *Hin*dIII were separated and blotted onto a nylon membrane. The membrane was probed with the digoxygenin-labeled cDNAs for genes expressed in pollen, washed, and visualized as described by Ushijima *et al.* (2001). An RNA blot analysis also was conducted as described by Sassa *et al.* (1997).

Cleaved amplified polymorphic sequence and RT–PCR/cleaved amplified polymorphic sequence: Genomic DNAs of Japanese pear cultivars were used as templates for PCR amplification of *SFBB* genes. The PCR products were digested with restriction enzymes to detect specific cleaved amplified polymorphic sequence (CAPS) bands. The primers and enzymes are listed in supplemental Table 2 at http://www.genetics.org/supplemental/. A CAPS analysis of the *S-RNase* genes also was conducted using the method described by Takasaki *et al.* (2004).

RNAs from the leaf and the floral organs of apple "Sekaiichi" and Japanese pear "Kosui" were treated with DNaseI (Nippongene). Their cDNAs were synthesized by SuperScript II (CLONTECH, Palo Alto, CA) with an oligo(dT) primer. The resultant cDNAs were used as templates for PCR amplification with gene-specific primers, and PCR products were treated with restriction enzymes to detect target-specific CAPS. A PCR was performed with *ExTaq* (TaKaRa), using a program of 30 cycles at 94° for 30 sec, 53° for 30 sec, and 72° for 45 sec and an initial denaturing of 94° for 2 min 30 sec and final extension of 72° for 7 min. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Identification of the most variable sites in the amino acid sequences: Amino acid sequences of 10 *SFBB* genes from apple and Japanese pear were aligned using the ClustalX program (Thompson *et al.* 1997) and manually adjusted. On the basis of the alignment, a normed variability index (NVI) was calculated for each site (Kheyr-Pour *et al.* 1990). Sites with an NVI > -0.25 were identified as the most variable sites.

Calculation of K_a- and K_s-values: DNA sequences were aligned using GENETYX-MAC (version 13; Software Development, Tokyo). After gaps were removed, a codon-by-codon alignment was carried out manually. On the basis of the alignment, DNAsp (Rozas *et al.* 2003) was used for the calculation of K_a- and K_s-values.

RESULTS

Construction of BAC contigs for the apple *S* locus: A BAC library from the apple cultivar Florina (Vinatzer *et al.* 1998) was screened using S^9-RNase cDNA as a probe. Of the five clones obtained, three contained an S^9-RNase gene and two included an S^3-RNase gene. For the S^9 haplotype-derived clones, overlapping BAC clones were further screened. End-sequence probes derived from the initial BAC clones produced smear patterns on apple genomic DNA blots, suggesting that they contained repetitive sequences and were not suitable

Figure 2.—Amino acid sequence alignment of the *S* locus F-box genes. Amino acid sequences of *SFBB* genes and other *S* locus F-box genes were aligned using ClustalX. Abbreviations of species are listed in Tables 2 and 3. Conserved sites and relatively conservative sites are marked with asterisks and dots, respectively. Double-headed arrow shows the F-box region.

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PiSLF2              1 MANGILKKLPEDLVFLILLTFPVKSLMRFKCISKAWSILIQSTTFINRHINRKTN---TKDEFILFKRAIK-----DD---EEEFINILSFFSGHVDVLN     89
PdSFBa              1 MT--FTPRKKE-ILIDILVRLPAKSLVRFLFTCKVWSDLISSSSFVSTHLNRNVTKHAHVYFLCFHHPNFECLVDPDDPCFEQELQWSLFSYETFEQCSE     97
PdSFBb              1 MT--FTLRKKE-TLIDILVRLPAKSLVRFLCTCKSWMDLIGSSSFVSTHLHRNVTKHAHVSLLCLHHQSFECQVDPDDPYVGQELQWSLFCDETFVLCSK     97
PdSFBc              1 MT--FKLGKKE-ILIDILARLTAKSLVRFLCTCKSWSDLIGRSSFVSTHINRNVTKHAHVYLLCLHHPTFERQNDNDDPFVEEELLWSLFSNETFEQFSK     97
PdSFBd              1 MT--FALRKKE-ILIDILVRLPAKSLVRFLCTCKSWSDLIGSSSFVGTHLHRNVTGHAQAYLLCLHHPNFECQRDDDDPYFKEELQWSLFSNVTFEESSK     97
PdSLFc              1 M-------WEEMALRHILPRLPSKSLMRFKCVRKSWYTLINNPTFVENHLSNSMQSKLS--TCVLFSRFVQSDANSDE--KELAFSFIYLRNDYDDDEHN     89
PdSLFd              1 M-------WEEMALRHILPRLPSKSLMRFKCVRKSWYTLINNPTFVENHLSNSMQSKLS--TCVLFSRFVQSDANSDE--KELAFSLLYLRNDYDDDEHN     89
PmSLFL1-S7          1 M-------WEEMALRHILPRLPSKSLMRFKCVRKSWYTVINNPTFVENHLSNSMHNKLS--TCILVSRFVQSDTNSDE--KELAFSFLHLRNDYDDAEHN     89
MdSFBB3-alpha       1 MSHVRESETPEDRVVEILSRLPPKSLMRFKCIHKSWFSLINNLSFVAKHLSNSVDNKLSSSTCILLNRSQAHIFPDQS--WKQEVFWSMINFSIDSDENN     98
MdSFBB3-beta        1 MSQVHESETPEDKVVEILCRLPPKSLMRFKCIRKSWCTLINRPSFVAKHLNNSVDNKLSSSTCILLNRSQAHIFPDQS--WKQEVPWSTINLSIDSDEHN     98
MdSFBB9-alpha       1 MSQVRESETPEDQVVEILSRLPPKSLMRFKCIRKSWCTIINSSSFVAKHLSNSIDNKLSSSTGILLNRCQVHVFSDRS--WKQDVFWSMINLSIDSDKNN     98
MdSFBB9-beta        1 MSQVRESETLEDKVVEILSKLPPKSLMRFKCIRKSWCTIINSPSFVAKHLNNSMDYKLSSTTCILLNRCQVHVFPDRS--WKQDVFWSMINLSIDSDEHN     98
PpSFBB4-alpha       1 MSQVHESETPEDKVVEILSRLPPKSLIRFKCVRKSWCTIINSPSFVAKHLSNTVDNKFSSFTCILFNRSQVHVFADRS--WKRDVFWSMINLSIESDEHN     98
PpSFBB4-beta        1 MTQVRESETPEDRVAEILSRLPPKSLMRFKCIRKSWGTIINNPSFMAKHLSNSVDNKFSSSTSILLHRSQMPVFPDRS--WKREYFWSMINLSHDSDEYN     98
PpSFBB4-gamma       1 MSQVRESETPEDRMVEILSRLPPKSLMRFKCIRKSWCTLINSPCFVAKHLSDSVDNKLSSSTCILLNCSQAHVCSEKS--WKQEVSWSVINLSIDGDE- -     96
                      *  . .... ..   .** ....***.**.. .*.* ..*....*...*..... . .. ....  .        .  . . .  ..  . . . .. .
                      <------------------------------------------------>
                          F-box

PiSLF2             89 PLFPDMDVS------YMTSKCDCTPNPLIGPCDGLIALTDT-----IITIVLNPATRNFRVLPASPFGCPKGY-------HRSVEGVGFGLDTISNYYKV    171
PdSFBa             97 LSHPLGSPEPY---------------RIYGSTNGLICISDAILSLESPIHIWNPSVRKLRTLPMTTN-NIEFS----------YIDLHFGFHPGVNDYKA    171
PdSFBb             97 LSHPLGSTEQYY--------------GIYGSSNGLVCISDEILNFDSPIYIWNPSVRKLRTPPLSTNINIKFS----------HVALQFGFHPGVNDYKT    173
PdSFBc             97 LSNPLGSTEHY--------------GIYGSSNGLVCISDEILNYDSPIHIWNPSVRKFRTPPISPN-NIKFV----------YVALQFGFHSGVNDYKA    171
PdSFBd             97 LSHPLGSTEHY--------------VIYGSSNGLVCISDEIMNFDSPIHIWNPSVKKLRTTPISTSINIKFS----------HVALQFGFHSGVNDYRA    172
PdSLFc             89 LNFVVEDIKFPLSSGQFIGLEDVESPSILGHCNGIVCLSPCS----DNLVLCNPAIKEIKLLPKSGLPDWWG-----------CAVGFGYDPKSKDYKV    173
PdSLFd             89 VNFVVEDIKFPLSSGRFIGLEDVESPSILGHCNGIVCLSPCS----DNLVLCNPAIKEIKLLPKSGLPDWWG-----------CAVGFGYDPKSKDYKV    173
PmSLFL1-S7         89 LNFVVEDIKFPLSSGRFIGLEDVESPSILGHCNGIVCLSPCS----DNLVLCNPAIKEIKLLPKSGLPDWWG-----------CAVGFGYDPKSKDYKV    173
MdSFBB3-alpha      98 LHYDVEDLN------IPFALKDHDFVLIFGYCNGILCVEAG-----KNVLLCNPATREFKQLPDSCLLLPSPP-ERKFELETNFQALGFGYDCNAKEYKV    186
MdSFBB3-beta       98 LHYDVEDLI------IPFPLEDHDFVLIFGYCNGIVCVDAG-----KNVLLCNPATREFRQLPDSCLLQP-PP-KGKFELETTFQALGFGYDCNAKEYKV    185
MdSFBB9-alpha      98 LYYDVEDLN------IPFPMEDQDNVELHGYCNGIVCVIVG-----KNVLLCNPATGEFRQLPDSSLLLPLP--KGRFGLETVFKGLGFGYDCKAKEYKV    185
MdSFBB9-beta       98 LHYDVEDIN------IPFPMEDQDNVELHGYCNGIVCVIVG-----KNVLLCNPATGEFRQLPNSPLLLPLP--KGRFGLETTFKGMGFGYDCKSKEYKV    185
PpSFBB4-alpha      98 LDYDVEDLN------IPFPMEVQDNVQLYGYCNGIVCVIVG-----ENVLLCNPATREFKQLPDSSLLLPLP--TGKFGLETLFKGLGFGYDCKTKEYKV    185
PpSFBB4-beta       98 LYYDVEDLN------IQFPLEDHDHVSIHGYCNGIVCLIVG-----KNAVLYNPATRELKQLPDSCLLLPSPP-EGKFELESTFQGMGFGYDSKAKEYKV    186
PpSFBB4-gamma      96 LHYDIEDLTI-----VPFLKDGPHEVEIHGYCDGIVCVTVD-----ENFFLCNPATGEFRQLPDSCLLLPLPGVKEKFGLETTLKGLGFGYDCKAKEYKV    186
PpSFBB5-alpha      98 LHYDVKDLN------IPFPMEVQDNVQLYGYCNGIVCVIVG-----ENVLLCNPATREFKQLPDSSLLLPLP--TGRFGLETLFKGLGFGYDCKTKEYKV    185
PpSFBB5-beta       98 LYYDVEDLN------VQFPLEDHEHISVHGYCNGIVCLIVG-----KNALLYNPATRELKQLPDSCLLLPSPP-EGKFELESTFQGMGFGYDSKAKEYKV    186
PpSFBB5-gamma      96 LHYDIEDLTI-----VPFLKDGPHEVEIHGYCDGIVCVTVD-----ENFFLCNPATGEFRQLPDSCLLLPLPGVKEKFGLETTLKGLGFGYDCKAKEYKV    186
                      . .....          .  ..     . *...*...           .  ..**........* . . . .    . ..     ..**.. . ..*..

PiSLF2            171 VRISEVY-CE-----EADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYW----VPCAGMLYKEMVHWFATTDMS----------MVILCFDMSTEMF    250
PdSFBa            171 VRMMGI---------------DKDAFAVEIYSLSTDSWKRIEVIPPWLKCD---WQHYKGIFLNGVVYHLIEKGPTFS----------LMSFDSGNAEF    242
PdSFBb            173 VRMMRT---------------NKSALAVEVYSLRTDCWKMIEAIPPWLKCT---WRHHKGTFLNGVAYHIIEKGPIFS----------IMSFNAGTEEF    244
PdSFBc            171 VRMMRS---------------NKDTFAVEVYSLRTDSWKMIETIPPWLKCT---WQHHTGTFFNGAAYHIIEKGPLFS----------IMSFDSGSEEF    242
PdSFBd            172 VRMLRT---------------NQNALAVEIYSLRTDSWTMIEAIPPWLKCT---WQHHQGTFFNGVAYHIIEKGPTFS----------VISFDSGSEEF    243
PdSLFc            173 SRIASY---------QVEIDGLIPPPRVEIYSLSTDSWREIKNNSLETDTT-CFFPDYFQMYFQGICYWVGYEQPKQSVEYEDEEQKPMVIFFDTGDEIF    263
PdSLFd            173 SRIASY---------QAEIDGLIPPPRVEIYTLSTDSWREINNNSLETDST-CFFPDYFQMYFQGICYWVGYEQPKQSVEYEDEEQKPMVIFFDTGDEIF    263
PmSLFL1-S7        173 SRIASY---------QAEIDGLIPPPRVEIYTLSTDSWREINNNSLETDST-CFFPDYFQIYFQGICYWVGYEQPKQSVEYEDEEQKPMVIFFDTGDEIF    263
MdSFBB3-alpha     186 VRIIEN--CEYSDDERTYYYRIALPHTAELYTTTANSWKEIKIDISSTTYS-----CSRSVFMKGFCYWYATDGEE-----------YILSFDLGDDTF    267
MdSFBB3-beta      185 VRIVEN--CEYSDDEQTFYHRIALPHTAEVYTTAANFWKEIKIDISIKTYH-----CSCSVYLKGFCYWFASDNEE-----------YILAFYLGDETF    266
MdSFBB9-alpha     185 VRIIENCDCEYSEGEESYYERILLPHTAEVYTMTADSWKEIKIDVSSDTDP-YCIPYSCSVYLKGFCYWFACDNGE-----------YIFSFDLGDEIF    272
MdSFBB9-beta      185 VRIIENCDCEYSDDGESYYERILLPHTAEVYTMTANSWKEIKIDISIETRW-YCIPYSGSVYLNGFCYWFAYDNGE-----------YVFSFDLGDEIF    272
PpSFBB4-alpha     185 VRIIENCDCEYSEGKESYYERILLPYTAEVYTTAANSWKEIKIDTSSDTDP-YCIPYSCSVYLKGFCYWFANDNGE-----------YIFSFDLGDEIF    272
PpSFBB4-beta      186 VKIIEN--CEYSDDMRTFSHRIALPHTAEVYVTTTNSWRVIEIEISSDTYN-----CSCSVYLKGFCYWFASDDEK-----------YVLSFDLGDEIF    267
PpSFBB4-gamma     186 VRIIDNYDCEYSEDGETYIEHIALPYTAEVYTMAANSWKEITIDILSKILSSYSEPYSYSVYLKGFCYWLSCDVEE-----------YIFSFDLANEIS    274
PpSFBB5-alpha     185 VRIIENCDCEYSEGKESYYERILLPYTAEVYTTAANSWKEIKIDTSSDTDP-YCIPYSCSVYLKGFCYWFANDNGE-----------YIFSFDLGDEIF    272
PpSFBB5-beta      186 VKIIEN--CEYSDDERTFSHRIALPHTAEVCITTTNSWRVIEIEISSDTYN-----CSCSVYLKEFCYWFASDDEE-----------CILSFDLGHEIF    267
PpSFBB5-gamma     186 VRIIDNYDCEYSEDGETYIEHIALPYTAEVYTMAANSWKEITIDILSKILSSYSEPYSYSVYLKGFCYWLSCDVEE-----------YIFSFDLANEIS    274
                      ...  .  ....          . .. ......  . .*... .             . ..........   .                . .*.......

PiSLF2            251 HDMKMPDT---------CSRITHELYYG-LV--ILCESFTLIGYSNPISSTDPA-HDKMHIWVMMEYGVSES--------WIMKYTIRP-LSIESPLAVW    328
PdSFBa            243 EEFITP--------------GAICRSRG-LFIAVYKEQICLL-FDFYCCEEEG--MEKIDFWVLEEKQWTQLAPFVYPS--DSYKIIG--ISIDNELLLR    320
PdSFBb            245 QEFIAP-------------DAVCSLWE-LRIDVYKENICLL-LDLYPSEEDG--MEKIDLWVLQEKRWKQLCPFIYPS-DHYYRTIG--MSMDNKLLMP    323
PdSFBc            243 EEFIAP-------------DAICNSRG-LCIDAYKDQICLL-FRFYGCEEEG--MRKVDLWVLQEKRWKQLCPFIIP--WKYCRTIG--TSIDNELLLQ    320
PdSFBd            244 EEFIAP-------------DAICSLWR-LCIHVYKEQICLL-FGYYSCEEEG--MENIDLWVLQEKRWKQLCPFIYDPLDDFYQIIG--ISTDNKLIMA    323
PdSLFc            264 NHILFPDSFYMYEEGSSYAYEMSYLMYCDLRIILWNGSIALFGFNRFSVFPDS-----YGVWVLDDFDGAKGS-------WTKHLTFEPLMGIKRVLEFW    351
PdSLFd            264 HNLLFPDSFYMYEEGSSYAYEMSYLMYCDLRIILWNGSIALFGFNRFSVFPDS-----YGVWVLDDFDGAKGS-------WTKHLTFEPLMGIKRVLEFW    351
PmSLFL1-S7        264 HNLLFPDSFYMYEEGSSYAYEMSYLMYCDLRIILWDGSIALFGFNRFSVCPDS-----YGVWVLDDFDGAKGS-------WTKHLNFEPLMGIKRVLEFW    351
MdSFBB3-alpha     268 HIIQLP-----------SRRESGFRFYY-IF--LRNESLASF-CSRYDRSEDS---ESCEIWVMDDYDGDKSS-------WTKLLNIGPLQGIKKPLTFW    342
MdSFBB3-beta      267 HIIQLP-----------SRRESGFTFDY-IF--LRNESLASF-CSPYNPSEDS---KLFEIWVMDDYDGVSSS-------WTKLLTVGPFKGVEYPLTLW    341
MdSFBB9-alpha     273 HIIELP-----------SRREFGFKFYG-IF--LYNESITSY-CSRYE--EDC---KLFEIWVMDDYDGVKSS-------WTKLLAVGPFKDIDYPLTLG    345
MdSFBB9-beta      273 HKIDLP-----------SRRESDFKFYG-IF--LYNESVTSY-CYRHE--EDC---ELFEIWVMDDYDGVKSS-------WTKLLTIGPLKDIDYPLTLW    345
PpSFBB4-alpha     273 RRIELP-----------FRRESDFNFYG-LF--LYNESVASY-CSRYE--EDC---KLLEIWVMDDHDGVKSS-------WTKLLTVGPFKDIESPSTFW    345
PpSFBB4-beta      268 HRIQLP-----------YRKESGFLFYD-LF--LYNESIASF-CSHYD-NDNAGILEILEIWVMDDCDGVKSS-------WTKLLTLGPFEDNENLLTFW    344
PpSFBB4-gamma     275 DMIELP-----------FRGEFGFKRDG-IF--LYNESLTYY-CSSYE--EPS---TLFEIWVMDYDDGFKSS-------WTKHLTAGPFIDMEFPLTPW    347
PpSFBB5-alpha     273 RRIELP-----------FRRESDFNFYG-LF--LYNESVASY-CSRYE--EDC---KLLEIWVMDDYDGVKRS-------WTKLLTVGPFKDIESPSTFW    345
PpSFBB5-beta      268 HRIQLP-----------CRKESGFLFCD-IF--LYNESIASF-CSHYDESDNSGILKILEIWVMDDCDGVKSS-------WTKLLTLGPFKGNENLLTFW    345
PpSFBB5-gamma     275 DMIELP-----------FRGEFGFKRDG-IF--LYNESLTYY-CSSYE--EPS---TLFEIWVMDYDDGFKSS-------WTKHLTAGPFTDMEFPLTPW    347
                        . .*           . .  .  .  ..  ......   .. .  ..       ..**... .. ...       ... .. ..   .   .. .

PiSLF2            329 KNHILLLQCRSGLLISYDLNSGEAKELNLHGFPDS----LSVKVYKECLTSIPKGSEYSTKVQKF                                      389
PdSFBa            321 KHDFSSVGLADLYLCNYESKQVRQAGIKLAVMEYGHHELFFATTYIESLLFLNKSLKRDV-----                                      380
PdSFBb            324 RRDYT-SGIADLHLCDYESKQVLETGIKLAVMKYGEIEFLFSISYIESLVLLNNY----------                                      377
PdSFBc            321 KKDFN-KGAADLCLCNYESKQVLETGIKLAIMTYGEIDFLFSFTYIESLVLLNNY----------                                      374
PdSFBd            324 RKDFS-KGGVELQLGNYESKQVLETGIKLAIMRYGESEFLFAMTYVESLALLNN-----------                                      376
PdSLFc            352 RSDEILMVTEDGDIVSYNLATQKLENLPMNSLSD-----FETIVYVNSLVSITRGNKLESVDI--                                      409
PdSLFd            352 KSDEILMVTIDGDIVSYNLDTEKLKNLPMNIPSD-----FETIVHVNSLVSITGGNKLESVDI--                                      409
PmSLFL1-S7        352 RSDEILMVTEDGDIVSFNLAIEKLKNLPMKIPSD-----FETIVYVNSLLPIT------------                                      399
MdSFBB3-alpha     343 RSDELLMLDSDGRATSYNYSTRNLKYLHIPPILNRVV-DFEVLIYVKSIVHVK------------                                      394
MdSFBB3-beta      342 KCDELLMLASDGRATSYNSSTGNLKYLHIPPILNKVV-DFEGLIYVKSIVPLK------------                                      393
MdSFBB9-alpha     346 KFDEVLMLGSYGRAAFCNSSTGNLKYLHIPPIIN------WMIDYVKSIVPVK------------                                      392
MdSFBB9-beta      346 KCDEVLMLGSYGRAASCNSSTGNLRYLHIPPIIK------WMMDYVKSIVPVQ------------                                      392
PpSFBB4-alpha     346 KCDEVLILSSYGKATSYNSGTENLKYLHIPPIIN------WMIDYVETIVPVK------------                                      392
PpSFBB4-beta      345 KSDELLMVTSDKRAISFNSSTGNLKYIHIPPIMNKVT-DFEALIYVESFVSVK------------                                      396
PpSFBB4-gamma     348 KRDELLMIASDGRAASYNSCTGNFKYLHIPVIINQN----RVVDYVKSIILVN------------                                      396
PpSFBB5-alpha     346 KCDEVLILSSYGKATSYNSSTGNLKYLHIPPIIN------WMIDYVETIVPVK------------                                      392
PpSFBB5-beta      346 KSDELLIVTSDQRAISYNSSTGNRKYIHIPPIINKIT-DLEALIYVESIVSIK------------                                      397
PpSFBB5-gamma     348 KRDELLMIASDGRAASYNSCTGNFKYLHIPVVINQN----RIVDYVKSIILVN------------                                      396
                      . .. ..  ... . .... . ........             .. . .
```

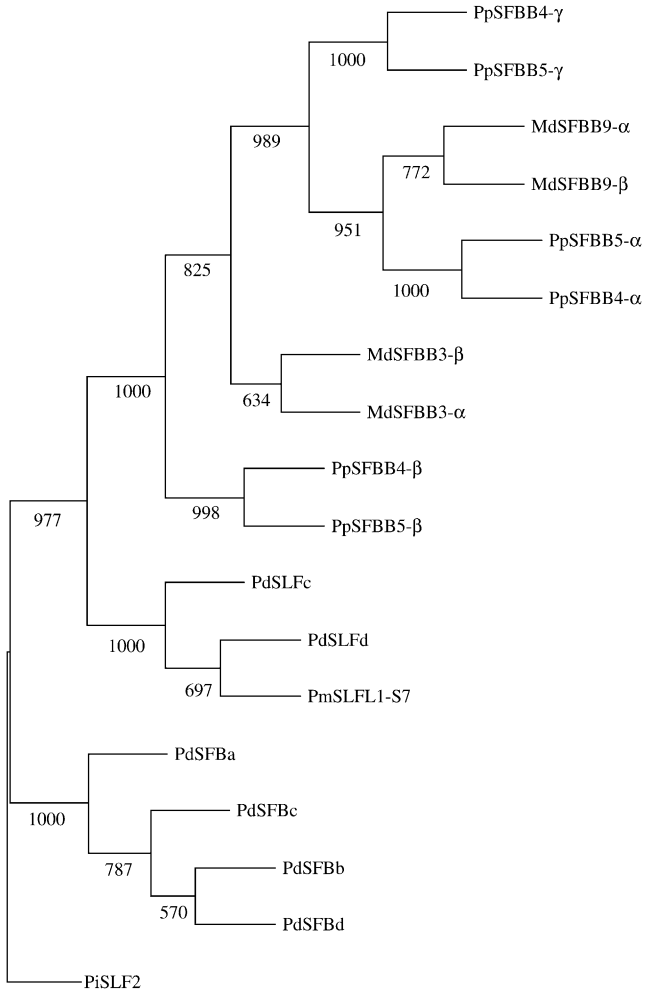

FIGURE 3.—Neighbor-joining tree of the *S* locus F-box genes with 1000 bootstraps.

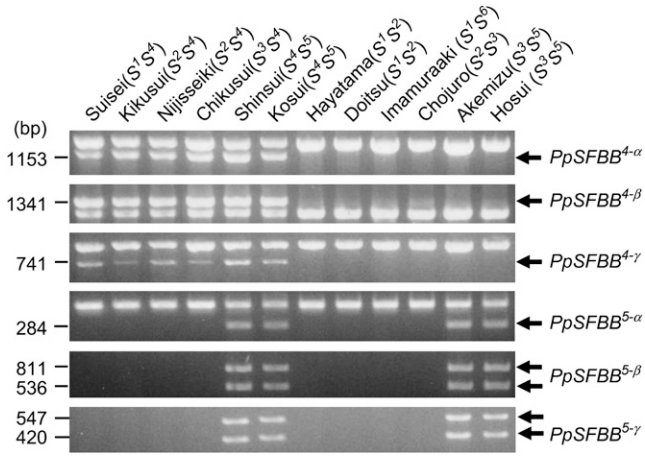

FIGURE 4.—*S*-haplotype-specific sequence polymorphism of Japanese pear *SFBB* genes. Japanese pear cultivars with different *S* genotypes were subjected to CAPS analysis for *SFBB* genes.

for library screening. The BAC clones were then subjected to shotgun sequencing, and the draft data were used to select candidate probes to identify further BAC clones. The library was screened with probes that produced single bands on genomic DNA blots. Ultimately, a BAC contig consisting of seven overlapping clones corresponding to the S^9 haplotype was constructed. A schematic representation of the BAC contig is shown in Figure 1a.

Analysis of a 317-kb sequence of the S^9 haplotype identified multiple, related F-box genes from the apple *S* locus: Of the seven BAC clones in the S^9-haplotype contig, three clones (90A15, 34G16, and 45M19) were completely sequenced. The entire 317-kb sequence contains a 169-kb region upstream of the S^9-RNase gene and a 148-kb region downstream of the S^9-RNase gene. The 317-kb sequence was annotated by the *R*ice *G*enome *A*utomated *A*nnotation *S*ystem (RiceGAAS) (SAKATA *et al.* 2002; http://ricegaas.dna.affrc.go.jp/index.html), which automatically analyzes large sequences by using several programs for prediction and analysis of protein-coding sequence, for example, Blast (ALTSCHUL *et al.* 1990), AutoPredLTR (SAKATA *et al.* 2002), and GENSCAN (BURGE and KARLIN 1997).

GENSCAN identified 71 open reading frames (ORFs) (Table 1). Of the 71 ORFs, 27 are homologous to retrotransposons, 36 show no significant homology to sequences in the databases, and 3 are similar to hypothetical proteins of rice, Medicago and Arabidopsis. Five ORFs—ORF20, ORF29, ORF43, ORF61, and ORF63—show homology to known genes. ORF43 was identified as the S^9-RNase gene, and ORF63 was shown to be homologous to a putative aminotransferase of rice. ORF20, ORF29, and ORF61 are homologous to Prunus *SLFL1*, a monomorphic F-box gene found in the *S* locus (ENTANI *et al.* 2003; USHIJIMA *et al.* 2003). ORF29 and ORF61 were named *MdSFBB*$^{9\text{-}\alpha}$ and *MdSFBB*$^{9\text{-}\beta}$ (*S* locus *F-b*ox *b*rothers of *M. domestica*), respectively. *MdSFBB*$^{9\text{-}\alpha}$ and *MdSFBB*$^{9\text{-}\beta}$ are located 42 kb upstream and 93 kb downstream of the S^9-RNase gene, respectively (Figure 1a). Although ORF20 is homologous to *SLFL1* and to the *MdSFBB* genes, the predicted amino acid sequence of 87 amino acid residues is much shorter than that of the *SFBB* genes (392 aa). Furthermore, while the downstream sequence of the stop codon for ORF20 is also homologous to *MdSFBB* genes, it contains several indels including a 1.4-kb insertion of unknown sequence; thus, ORF20 was considered to be a pseudogene and named Ψ*MdSFBB*$^{9\text{-}\alpha}$. Ψ*MdSFBB*$^{9\text{-}\alpha}$ showed 84.0 and 83.3% nucleotide identity to *MdSFBB*$^{9\text{-}\alpha}$ and *MdSFBB*$^{9\text{-}\beta}$, respectively. Similarly, at a position ~3 kb downstream of *MdSFBB*$^{9\text{-}\beta}$, another pseudogene was identified, Ψ*MdSFBB*$^{9\text{-}\beta}$. Ψ*MdSFBB*$^{9\text{-}\beta}$ contained several indels including a 980-base insertion of unknown sequence. Ψ*MdSFBB*$^{9\text{-}\beta}$ showed a 75.5 and a 78.1% nucleotide identity to *MdSFBB*$^{9\text{-}\alpha}$ and *MdSFBB*$^{9\text{-}\beta}$, respectively. In addition, an ~800-base sequence with similarity to *SFBB* was found ~8.3 kb upstream of Ψ*MdSFBB*$^{9\text{-}\alpha}$. With

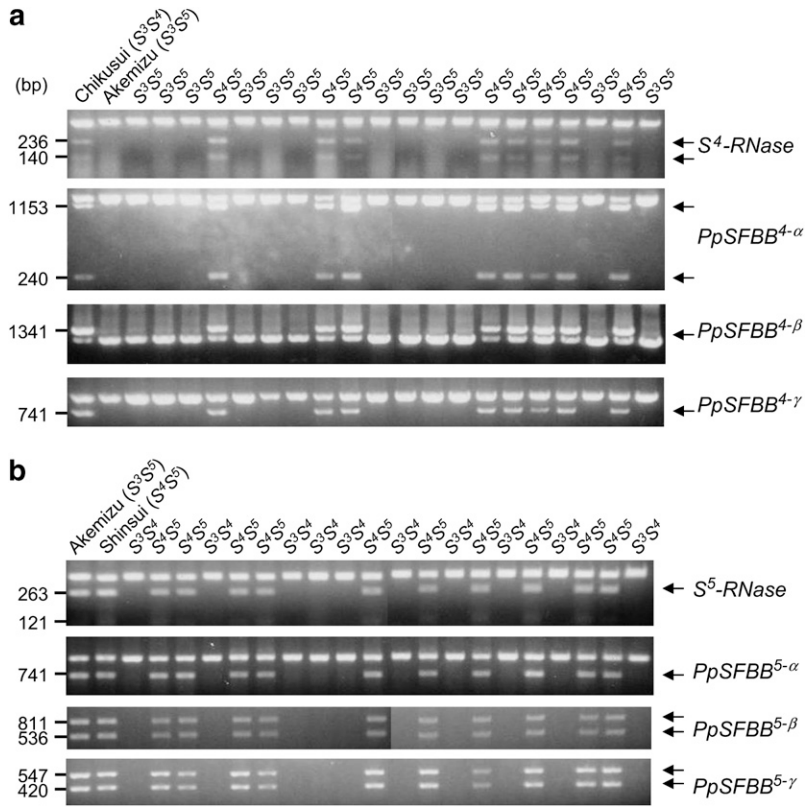

FIGURE 5.—Linkage analysis of *SFBB* genes and the *S-RNase* genes in Japanese pear. (a) Linkage between S^4-RNase and *PpSFBB*^{4} genes. (b) Linkage between S^5-RNase and *PpSFBB*^{5} genes. Parents (left two lanes) and their progenies were analyzed using CAPS.

the exception of the *SFBB* genes, no other F-box genes were found in the 317-kb sequence of the S^9-haplotype. *MdSFBB* genes are more homologous to Prunus *SLFL1* (34.4–37.0% amino acid identity) than they are to *SFB* (21.3–28.2% amino acid identity; Table 2).

***S*-haplotype-specific sequence polymorphism of *SFBB*:** Apple *SFBB* homologs were obtained using a PCR from the *S*^{3}*-RNase* gene containing two BAC clones, 66L6 and 72N11. The *SFBB* homolog sequences were related but not identical with each other, suggesting that they were derived from nonoverlapping regions of the two BAC clones. The two *SFBB* homologs were named *MdSFBB*$^{3\text{-}\alpha}$ and *MdSFBB*$^{3\text{-}\beta}$, respectively (Figure 1b).

An RT–PCR was used to clone pollen-expressed *SFBB* homologs from Japanese pear, using the primers derived from *MdSFBB* sequences. Six cDNAs were obtained from the Kosui (S^4S^5) pollen, a cultivar of Japanese pear: *PpSFBB*$^{4\text{-}\alpha}$, *PpSFBB*$^{4\text{-}\beta}$, *PpSFBB*$^{4\text{-}\gamma}$, *PpSFBB*$^{5\text{-}\alpha}$, *PpSFBB*$^{5\text{-}\beta}$, and *PpSFBB*$^{5\text{-}\gamma}$.

SFBB genes showed 58.4–99.0% deduced amino acid identity with each other (Table 3). While apple *SFBB* genes from the same haplotypes were more similar to other haplotypes, Japanese pear genes were more related to other haplotype genes of the same group (*i.e.*, α-, β-, and γ-groups) (Table 3, Figures 2 and 3, see below).

S-haplotype specificity was analyzed by correlating Japanese pear *S* genotype with *PpSFBB* gene polymorphism. Since the *SFBB* genes are similar to each other, a CAPS procedure was used to detect polymorphism. Amplification by group-specific primers was followed by digestion with a restriction enzyme to reveal gene-specific patterns. This CAPS analysis showed that *PpSFBB* genes are specific to their respective *S* haplotypes (Figure 4).

A CAPS analysis also was used to examine the linkage between *PpSFBB* and *S-RNase* genes. A segregating population derived from a cross between Chikusui (S^3S^4) and Akemizu (S^3S^5) was analyzed for a linkage between S^4-RNase and the *PpSFBB*^{4} genes. Figure 5 shows the representative results of this CAPS analysis. Three of the *PpSFBB*^{4} genes were detected specifically in the S^4-containing progenies (18 of 40 plants analyzed), suggesting a linkage between S^4-RNase and *PpSFBB*^{4}. A similar analysis of 40 progenies derived from a cross between Akemizu (S^3S^5) and Shinsui (S^4S^5) also showed a linkage between the *PpSFBB*^{5} genes and S^5-RNase.

***SFBB* genes are specifically expressed in the pollen:** Organ-specific expression of the *SFBB* genes was analyzed using RNA blotting. Pollen-specific signals were detected for all *SFBB* genes (Figure 6). Since the *SFBB* genes show relatively high homology with each other in some pairs as is the case with *SLF* (SIJACIC *et al.* 2004), these RNA blot results may suggest that each respective *SFBB* gene and/or its homologous gene(s) are specifically expressed in the pollen.

To determine whether each *SFBB* gene is specifically expressed in the pollen, an RT–PCR was performed in combination with a CAPS analysis (RT–PCR/CAPS).

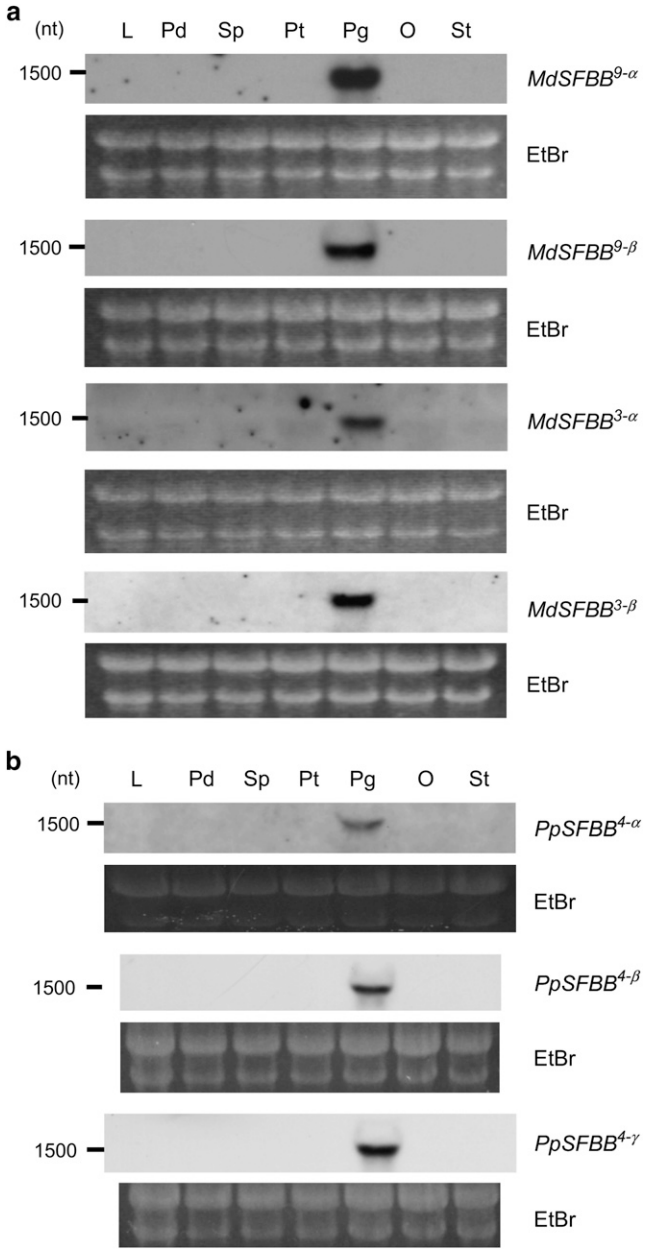

FIGURE 6.—RNA gel blot analysis of *SFBB* genes. (a) Apple *SFBB* genes. (b) Japanese pear *SFBB* genes. Lf, leaf; Pd, pedicel; Sp, sepal; Pt, petal; Pg, pollen grain; Ov, ovary; St, style.

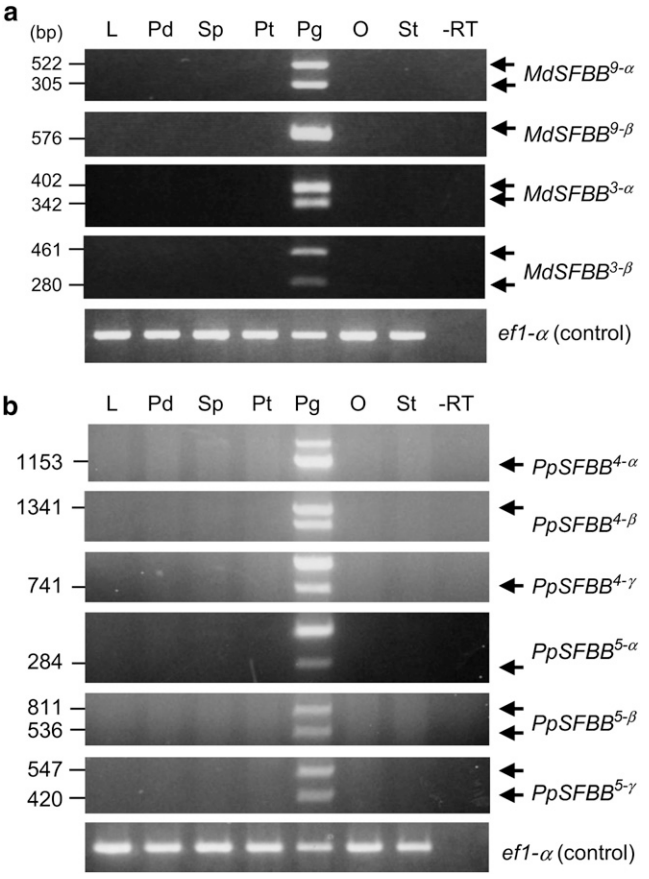

FIGURE 7.—RT–PCR/CAPS analysis of *SFBB* expression in different organs. (a) Apple *SFBB* genes. (b) Japanese pear *SFBB* genes. −RT, pollen grain RNA negative control experiment performed without reverse transcriptase.

cDNAs derived from different organs were subjected to CAPS analysis to detect target, sequence-specific restriction fragments. Results showed that all the *SFBB* genes are actually and specifically expressed in pollen (Figure 7).

The S^{4sm} haplotype, a style-specific self-compatible Japanese pear mutant, lacks the S^4-RNase-containing region and retains *SFBB*^{4} genes: We have previously analyzed a style-specific self-compatible Japanese pear mutant "Osa-Nijisseiki," which has a defective S^4 haplotype named S^{4sm}, and shown that the S^{4sm} haplotype lacks the S^4-RNase gene-containing region for at least 4 kb (SASSA *et al.* 1997). Since pollen that has the S^{4sm} haplotype is normally rejected by an S^4 pistil, it is expected that the S^{4sm} haplotype retains the pollen S^4 gene and that this gene is located outside the deletion region.

To ascertain whether *SFBB* genes are retained in the S^{4sm} haplotype, an ~130-kb cosmid contig for the normal S^4 haplotype was constructed and used to analyze the deletion region. Using probes derived from different positions on the contig, a genomic DNA blot analysis was conducted to determine if the corresponding region is deleted in the S^{4sm} haplotype. Many of the cosmid end probes displayed smear patterns on a genomic DNA blot, probably because of the repetitive sequences that are rich in the *S* loci (COLEMAN and KAO 1992; MATTON *et al.* 1995; USHIJIMA *et al.* 2001). Among the probes that displayed a single band on a DNA blot, the most upstream probe, 11-17Sph-L (located 37 kb upstream of *S^4-RNase*), showed nearly no signal in Osa-Nijisseiki, suggesting that the corresponding region is deleted in the S^{4sm} haplotype (Figure 8A). Similarly, the most downstream probe, 11-1R, detected nearly no signal in Osa-Nijisseiki. Faint signals found in Osa-Nijisseiki are derived from wild-type cells retained in the mutant, which was somaclonally derived from the original variety Nijisseiki (S^2S^4) and is chimeric for the S^4 haplotype (SASSA *et al.* 1997). Therefore, a >110-kb region, located

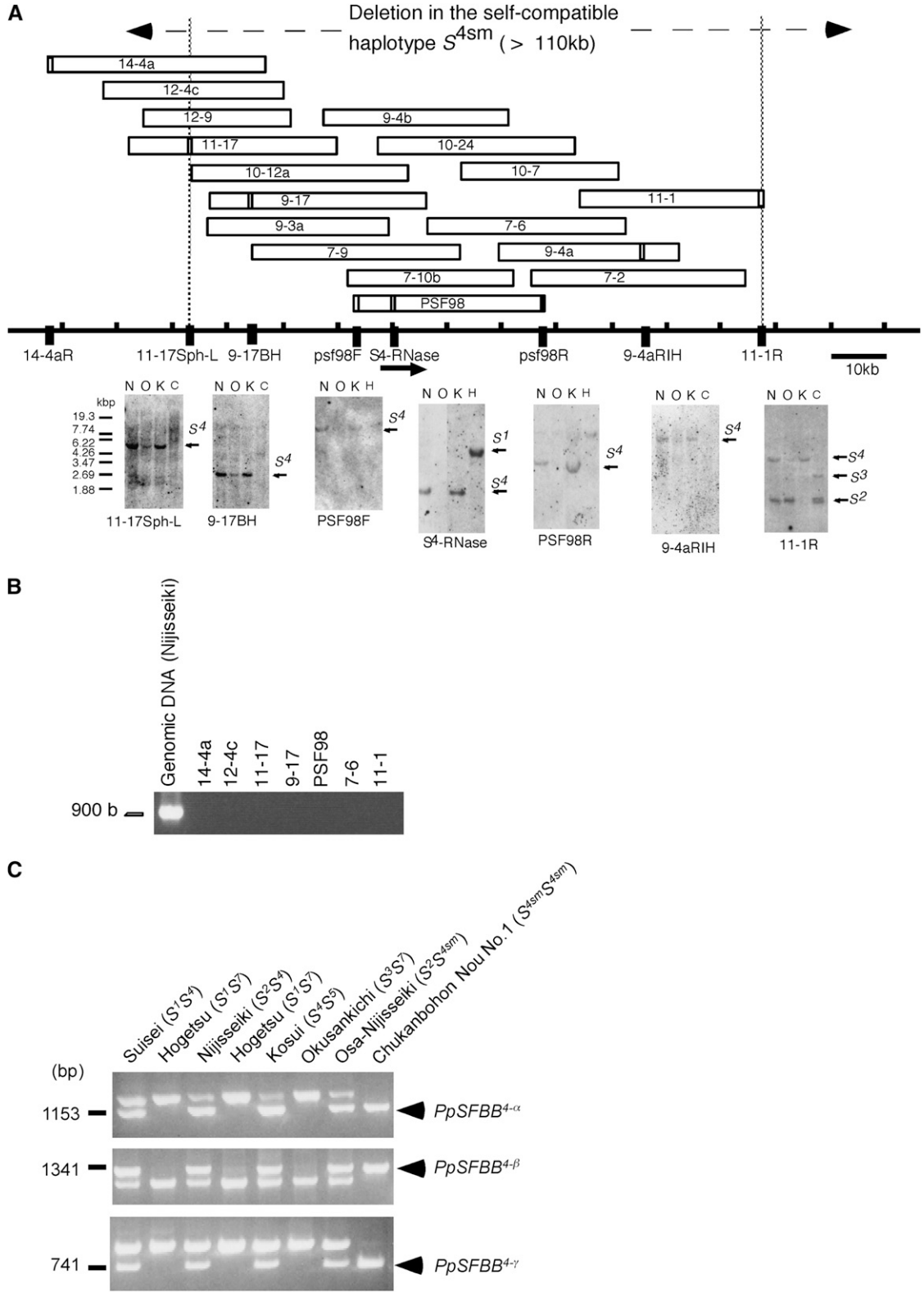

FIGURE 8.—Analysis of the style-specific, self-compatible haplotype S^{4sm}. (A) A cosmid contig for the S^4 haplotype and DNA gel blot analysis with cosmid-derived probes. Open boxes denote cosmid clones. N, Nijisseiki (S^2S^4); O, Osa-Nijisseiki (S^2S^{4sm}); K, Kosui (S^4S^5); C, Chojuro (S^2S^3); H, Hayatama (S^1S^2). (B) PCR amplification of *SFBB* genes from genomic DNA of Nijisseiki and cosmid clones for the S^4 haplotype. (C) CAPS analysis of the $S^{4sm}S^{4sm}$ genotype, Chukanbohon Nou No.1, and other *S* genotypes.

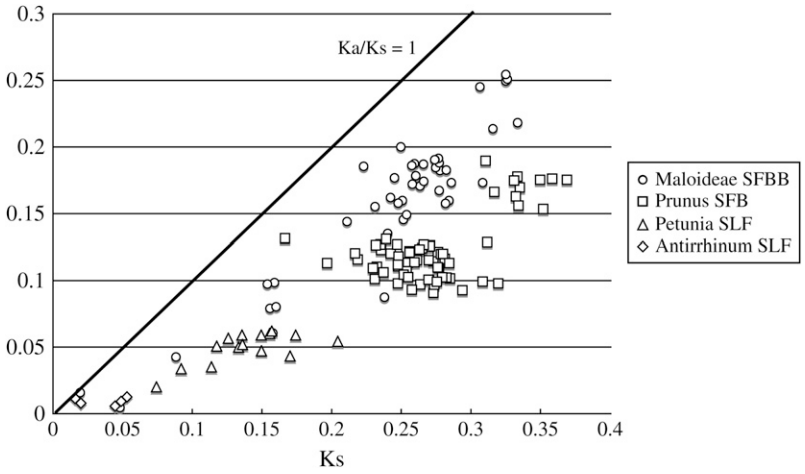

FIGURE 9.—Pairwise comparisons of synonymous (K_s) and nonsynonymous (K_a) substitution frequencies in the *SFBB* and other *S* locus F-box genes. Circles, squares, triangles, and diamonds denote the data for *SFBB* of Maloideae, *SFB* of Prunus, *SLF* of Petunia, and *SLF* of Antirrhinum, respectively.

between 11-17Sph-L and 11-1R, is deleted in the S^{4sm} haplotype.

Subsequently, we examined whether the *SFBB*^{4} genes are retained in the S^{4sm} haplotype. *SFBB* genes could not be amplified by PCR from the cosmid clones of the S^4 haplotype (Figure 8B). A subsequent CAPS analysis using an $S^{4sm}S^{4sm}$ homozygous genotype "Chukanbohon Nou No.1" did successfully amplify the *SFBB*^{4} genes from the $S^{4sm}S^{4sm}$ plant, indicating that the *SFBB*^{4} genes are retained in the S^{4sm} haplotype (Figure 8C).

Nucleotide substitution patterns of *SFBB* genes: For SI genes, new specificity is considered to have a reproductive advantage and tends to be maintained in a population. A sequence comparison has shown that SI genes have excess nonsynonymous substitutions, which supports the hypothesis that they are positively selected (NEWBIGIN and UYENOYAMA 2005). Consequently, we analyzed *SFBB* sequences to determine whether they show nucleotide substitution patterns similar to those of known SI genes.

We used a codon-by-codon alignment of *SFBB* sequences to calculate the ratio of the nonsynonymous substitutions per nonsynonymous site (K_a) to the synonymous substitutions per synonymous site (K_s), or K_a/K_s. A similar analysis also was conducted for Prunus *SFB*, Petunia *SLF*, and Antirrhinum *SLF*. Figure 9 shows the results of the pairwise comparison of the K_a/K_s-values. The average K_a/K_s-value for *SFBB* (0.69) was higher than the K_a/K_s-values for Prunus *SFB* (0.45), Petunia *SLF* (0.34), and Antirrhinum *SLF* (0.27) (Table 4). These values indicate that *SFB* and *SFBB* are more diverged than *SLF*. As whole molecules, the K_a/K_s-values for all the genes were <1 and were lower than the K_a/K_s-values for S-RNase's: Maloideae, 0.83; Prunus, 0.54; and Solanaceae, 0.75 (MA and OLIVEIRA 2002). This may be due, partly, to the larger size of the F-box genes compared to the *S-RNase* genes and, partly, to the limited region(s) critical for recognition. For genes involved in recognition systems such as SI and disease resistance, it is known that the portions related to recognition are under positive selection (ISHIMIZU *et al.* 1998; BERGELSON *et al.* 2001; IKEDA *et al.* 2004).

To detect diverged amino acid sites that may be important for recognition, an NVI (KHEYR-POUR *et al.* 1990) was calculated for the *SFBB* genes. Forty-eight variable sites were detected (supplemental Figure 1 at http://www.genetics.org/supplemental/). Four regions with particularly rich variable sites were named V1–V4 (Figure 10 and supplemental Figure 1). K_a/K_s-values were calculated for these four regions as well as for the F-box region. The K_a/K_s-values of the F-box, V1, V2, V3, and V4 regions were 0.22, 1.18, 1.33, 0.64, and 0.58, respectively (Table 5). These values suggest that V1 and V2 are under positive selection and that the F-box region is under purifying selection.

DISCUSSION

Organization of the *S* locus of Maloideae: Aiming to identify the pollen *S* gene in Maloideae, a subfamily of Rosaceae, we completely sequenced the 317-kb apple S^9 haplotype. This represents—along with the 328-kb

TABLE 4

K_a/K_s-values of the *S* locus F-box genes

	Maloideae *SFBB*	Prunus *SFB*	Petunia *SLF*	Antirrhinum *SLF*
K_s average (SD)	0.2315 (0.0606)	0.2648 (0.0413)	0.1392 (0.0330)	0.0337 (0.0168)
K_a average (SD)	0.1586 (0.0573)	0.1202 (0.0242)	0.0482 (0.0121)	0.0092 (0.0025)
K_a/K_s	0.6853	0.4539	0.3463	0.2717

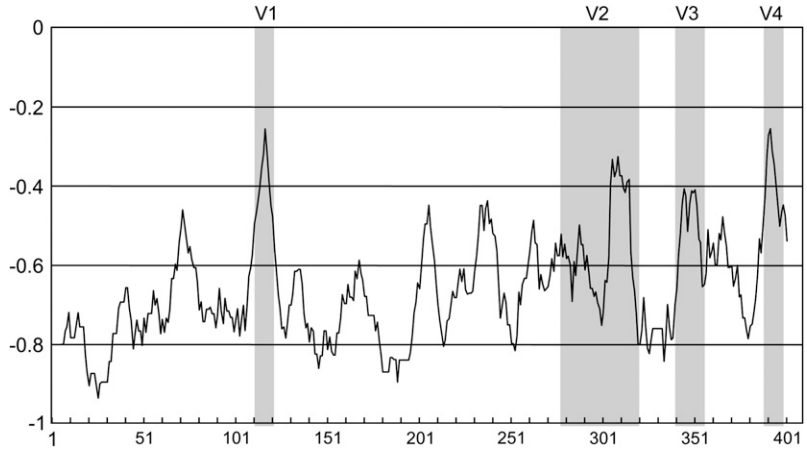

FIGURE 10.—Window-averaged plot of normed variability index at each site in the alignment of the *SFBB* genes. Variable regions are shaded.

Petunia S^2 haplotype (WANG *et al.* 2004)—one of the largest sequences for the *S* locus. In the 328-kb Petunia S^2 haplotype sequence, 31 ORFs showed high similarity to retrotransposons (WANG *et al.* 2004). Comparable numbers were found in the 317-kb apple S^9 haplotype: 27 of 71 predicted ORFs were homologous to retrotransposons. Although it has been suggested that the retrotransposon-rich organization of the Petunia *S* locus may reflect its centromeric location (WANG *et al.* 2004), there are no data showing the subcentromeric localization of the Maloideae *S* locus.

Several findings show that the Maloideae *S* locus is larger than the Prunus *S* locus. Analysis of the Japanese pear S^{4sm} haplotype showed that the pollen *S* gene must be located outside the deletion region by at least 110 kb. Additionally, the distances of $MdSFBB^{9\text{-}\alpha}$ and $MdSFBB^{9\text{-}\beta}$ from the S^9-RNase gene are 42 and 93 kb, respectively. In contrast, the distances between Prunus *S-RNase*'s and *SFB*s are 380 bases to 36 kb (YAMANE *et al.* 2003; USHIJIMA *et al.* 2004). Differences in the size of the *S* locus between species also have been reported in Brassica, a species with sporophytic SI. The *S* locus region of *Brassica oleracea* is much larger than that of *B. rapa* (FUJIMOTO *et al.* 2006). Expansion of the *S* locus region in *B. oleracea* has been partly attributed to the insertion of retrotransposons, which suggests higher retrotransposon activity in *B. oleracea* than in *B. rapa* (FUJIMOTO *et al.* 2006). It should be noted that Maloideae is considered to be of polyploid origin (EVANS and CAMPBELL 2002) and polyploidization can activate retrotransposons (MADLUNG *et al.* 2005). The abundant retrotransposons found in the apple *S* locus may help to prevent recombination at the chromosomal region and to maintain the tight linkage between S-RNase and the pollen *S* allele.

Related, multiple, polymorphic, and pollen-specific F-box genes in the *S* locus of Maloideae: Sequence analyses have revealed that the *S* loci of Prunus, Petunia, and Antirrhinum contain several F-box genes in addition to the pollen determinant *SFB/SLF* (LAI *et al.* 2002; ENTANI *et al.* 2003; USHIJIMA *et al.* 2003; WANG *et al.* 2004). In these species, however, each F-box gene is a single copy in a haplotype. In contrast, the *S* haplotypes of apple and Japanese pear contain two or three copies of the *SFBB* genes. During preparation of this article, CHENG *et al.* (2006) isolated *S* locus-linked and pollen-expressed F-box genes from apple by PCR and named them *SLF* of apple. The apple SLF was highly homologous to the SFBBs of apple and Japanese pear; however, CHENG *et al.* (2006) described a single *SLF* gene from each haplotype. *SFBB* genes, therefore, represent the first case of related and multiple F-box genes in the *S* locus. Initially, occurrence of multiple *SFBB* genes in a haplotype may appear inconsistent with the idea that they are the pollen determinant of GSI; the pollen-part factor F-box genes are single-copy genes in Prunus (*SFB*: ENTANI *et al.* 2003; USHIJIMA *et al.* 2003; YAMANE *et al.* 2003) and in Petunia (*SLF*: SIJACIC *et al.* 2004). However, apart from their multiplicity feature, *SFBB* genes are a good candidate for pollen *S* in Maloideae, as they show linkage to the *S-RNase* gene, *S* haplotype-specific sequence divergence, and

TABLE 5

K_a/K_s-values of F-box and variable regions of *SFBB* genes

	F-box	V1	V2	V3	V4
K_a (SD)	0.070 (0.025)	0.470 (0.293)	0.246 (0.108)	0.262 (0.121)	0.763 (0.593)
K_s (SD)	0.324 (0.149)	0.397 (0.239)	0.185 (0.073)	0.409 (0.246)	0.440 (0.266)
K_a/K_s	0.218	1.181	1.331	0.640	0.576
Pair no. of $K_a > K_s$ (Total 44)	1	21	30	12	9

pollen-specific expression. Unlike in Solanaceae, there is no report of subcentromeric localization of the *S* locus in Maloideae. Therefore, taking into consideration that Petunia *SLF*^{2} is located 161 kb from the S^2-RNase gene (Wang *et al.* 2004), it seems unlikely that another genuine pollen *S* gene is located outside the 317-kb region of the apple S^9 haplotype. Additionally, our analysis of the pistil-specific, self-compatible haplotype S^{4sm} showed that *PpSFBB*^{4} genes are retained in the S^{4sm} haplotype and are located outside the known deletion region. Taken together, these findings support the idea that the *SFBB* genes are the pollen *S* determinant in Maloideae.

An analysis of nucleotide substitution patterns of the *SFBB* genes and other pollen *S* genes showed that the *SFBB* genes have a higher average K_a/K_s-value than *SFB* of Prunus, *SLF* of Petunia, and *SLF* of Antirrhinum. Among the *S-RNase* genes, the K_a/K_s-value was also higher in Maloideae than in Prunus, suggesting that the *S-RNase* genes of Maloideae diverged more recently than those of Prunus (Ma and Oliveira 2002). The higher K_a/K_s-value of *SFBB* than of *SFB* may reflect the coevolution of the F-box and the *S-RNase* genes in Rosaceae. An amino acid sequence analysis of the SFBB detected four variable regions with high NVI values, and two of them (V1 and V2) were found to be under positive selection. Positive selection has been suggested in variable regions of *S-RNase* genes and *SFB* genes of Rosaceae, which supports the idea that these regions are critical for *S* specificity (Ishimizu *et al.* 1998; Ikeda *et al.* 2004). Positive selection detected in the variable regions of the *SFBB* genes is also consistent with the possible "self" recognition function of the protein. Although the K_a/K_s-values of the V3 and the V4 regions were <1, this may be due, partly, to high K_s-values in these regions and/or to gaps in the V4 region and may not exclude their potential importance in recognition.

The exceptional feature of *SFBB* as the pollen *S* candidate is its multiplicity. It is possible that only one *SFBB* gene in a haplotype is the pollen determinant. However, this seems unlikely, since pear shows competitive interaction (Crane and Lewis 1941; Lewis and Modlibowska 1942) and multiple *SFBB* genes with *S*-specific polymorphisms are expressed in pollen with normal GSI function. Expressed non-*S SFBB* genes may competitively interact with the pollen *S SFBB* to breakdown GSI in pollen. Another possibility is that all the expressed *SFBB* genes act together as the pollen determinant. Analyses of pollen-part, self-compatible mutations of Prunus have found that all mutations, both natural and X-ray-induced ones, were loss-of-function type (Ushijima *et al.* 2004; Sonneveld *et al.* 2005). However, loss-of-function mutations have not been reported in Maloideae, and pollen-part breakdown of GSI has been interpreted as a result of competitive interaction in tetraploid plants (Crane and Lewis 1941; Lewis and Modlibowska 1942). The occurrence of multiple pollen *S* genes also may explain the absence of deletion type of the pollen self-compatible mutation in Maloideae. However, sequence divergence of *SFBB* copies in a single haplotype may be unusual if the copies are only for backup function. Although duplication of a pollen *S* gene was reported for the S^b-haplotype of self-incompatible *Arabidopsis lyrata*, which exhibits sporophytic SI, sequences of the two copies of the *SCR*^{b} genes were identical to each other (Kusaba *et al.* 2001). An interesting possibility is that SFBB proteins form a multimer in pollen as suggested by Luu *et al.* (2001). However, other possibilities that only one of the *SFBB*s in a haplotype or none of them are involved in pollen *S* specificity cannot be excluded at present. Functional characterization of *SFBB* in pollen and a comparative analysis of the apple *S* locus structure with those of other species will shed light on the mechanism, variation, and evolution of the the S-RNase-based GSI system.

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