Identification and analysis of pollen *S* candidate genes in apple and pear

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Abbreviations

BAC	bacterial artificial chromosome
CAPS	cleaved amplified polymorphic sequence
cDNA	complementary DNA
dCAPS	derived cleaved amplified polymorphic sequence
DIG	digoxigenin
GSI	gametophytic self-incompatibility
HV	hypervariable
Ka	nonsynonymous substitutions per nonsynonymous site
Ks	synonymous substitutions per synonymous site
ORF	open reading frame
PCD	programmed cell death
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RiceGAAS	Rice genome automated annotation system
RNase	ribonuclease
RT	reverse transcription
SFB	*S* haplotype specific F-box gene
SFBB	*S* locus F-box brothers
SI	self-incompatibility
SLF	*S* locus F-box gene
SLFL	*S* locus F-box like
sm	stylar-part self-incompatibility mutant

General introduction

0.1 Apple and Pear Genomics

The plant family Rosaceae, comprised of over 100 genera and 3,000 species, is the third most economically important plant family in temperate regions (Dirlewanger et al. 2002). Rosaceae includes many important fruit, nut, ornamental, and wood crops. Most rosaceous crops have been bred by human intervention through sexual hybridization, asexual propagation, and genetic improvement since ancient times, 4,000 to 5,000 B.C. Rosaceae family has been traditionally classified into four subfamilies; Rosoideae (*Rosa*, *Fragaria*, *Potentilla*, and *Rubus*; x=7, 8, or 9), Amygdaloideae (*Prunus*; x=8), Spiraeoideae (*Spirea*; x=9), and Maloideae (*Malus*, *Pyrus*, and *Cotoneaster*; x=17; Potter et al. 2002). In recent years, representative crops such as apple (*Malus* spp.), peach (*Prunus* spp.), and strawberry (*Fragaria* spp.) have been studied well (Smartt and Simmonds 1995; Shulaev et al. 2008).

Apple (*Malus x domestica*) is one of the world's leading fruit crops widely grown with production in 2001 of almost 60 million tones. Japanese apple cultivar 'Fuji' is produced most and estimated to increase production in the future (O'Rourke et al. 2003). Thus, apple is very important crops not only in Japan but around the world. Most apple cultivars are diploid (2n=2x=34), and genome, 750Mb per haploid genome complement, is approximately the same size as that of tomato (*Solanum lycopersicum*; Tatum et al. 2005). Apple is model species in Rosaceae for the following reasons; i) There are many molecular markers (Shulaev et al. 2008). ii) *Agrobacterium*-mediated transformation method has established and developed in several laboratories (Bondt et al. 1996; Kotoda and Wada 2005). iii) Bacterial artificial chromosome (BAC) libraries were constructed (Venatzer et al. 1998, Xu et al. 2001). IV) A complete genome sequence of the apple is under way, and it will be completed by mid-2008 (Shulaev et al. 2008). Genomic research for pear (Japanese pear; *Pyrus pyrifolia*, European pear; *P. communis*) is alsoin progress. Map comparisons suggest that genome organization is conserved between apple and pear (Yamamoto et al. 2004).

Apple and pear trees are artificially pollinated by farmers or planted with other compatible cultivars in orchards, in order to make sure the fruit sets because these exhibit self-incompatibility (SI). Self-incompatibility research would contribute for fruits production and breeding programs. Apple is very useful plant material for studying self-incompatibility as described above. As well as in Japanese pear, cultivar 'Osa-Nijisseiki' is valuable for SI research. 'Osa-Nijisseiki' is staylar-part self-compatibility mutant, which lacks of pistil recognition function while pollen recognition function works (Sato et al. 1988, 1992). To analyze pollen *S* gene, this mutant is used in this study (see below).

0.2 S-RNase-based Self-incompatibility

Self-incompatibility is a genetic system that prevents self-fertilization by enabling the pistil to reject pollen from genetically related individuals, thus promoting outcrossing (de Nettancourt 2001). The specificity of the SI response is determined by the haplotyes of the polymorphic *S* locus, which contains at least one gene for the pistil determinant (pistil *S*) and one for the pollen determinant (pollen *S*). The families Rosaceae, Solanaceae and Plantaginaceae include species exhibit S-RNase-based gametophytic self-incompatibility (GSI).

Pistil *S* determinant, *S-RNase*, has been well characterized during the past two decades (Kao and Tsukamoto 2004). S-RNase protein and cDNA were firstly identified in *Nicotiana alata* by Bredemijer and Blass (1981) and Anderson et al. (1986), respectively. Then, it is confirmed that S-RNase has RNase activity *in vitro* (McClure et al. 1989). The role of *S-RNase* has been established via transgenic experiments in solanaceous plants (Lee et al. 1994, Murfett et al. 1994). The *S-RNase* was also determined as pistil *S* gene in the study of Japanese pear (Sassa et al. 1992, 1997) and apple (Sassa et al. 1994, 1996; Broothaerts 1995, 2004).

S-RNase has been studied in detail, while the nature of pollen *S* gene had been unknown for a long time. Recently, *SLF* (*S* locus F-box gene) and *SFB* (*S* haplotype specific F-box gene)

were identified as (putative) pollen *S* genes in the three families (McClure 2004). The identity of *SLF* as the pollen *S* determinant of *Petunia inflata*, a solanaceous species, was demonstrated by transformation experiment (Sijacic et al. 2004). In several *Prunus* species of Amygdaloideae, analysis of pollen-part self-compatible mutants revealed insertion/deletion or complete loss of *SFB* gene, suggesting that *SFB* is the pollen *S* (Ushijima et al. 2004; Sonneveld et al. 2005; Vilanova et al. 2006). However, attempts to isolate pollen *S* in Maloideae through a homology-based approach with *Prunus SFB* sequence information have been unsuccessful (Suzuki Y and Sassa H, unpublished results), most likely because of sequence diversity of pollen *S* between these subfamilies.

0.3 Overview of this study

This study is divided into three major parts. First, apple *S* locus was analyzed to obtain pollen *S* candidate gene. Around the region of the *S-RNase* was sequenced and its structural features were discussed (Chapter 1). Second, characterizations of isolated pollen *S* candidate genes (*SFBB*; *S* locus F-box brothers) were analyzed in apple and pear (Chapter 1 and 2). Finally, using *SFBB*$^{\gamma}$, one of the *SFBB* group, established new *S* genotyping system in Japanese pear (Chapter 3).

Chapter 1

Analysis of *S* locus and identification of pollen *S* candidates in Apple and Japanese pear.

1.1 Introduction

The S-RNase-based gametophytic self-incompatibility (GSI) system has been found in the families Solanaceae, Rosaceae, and Plantaginaceae. 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 (Goldraj 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 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 (Suzuki Y and Sassa H, unpublished results), most likely because of sequence diversity of pollen *S* between these subfamilies.

Here, I 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 I 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 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.

1.2 Materials and Methods

1.2.1 Plant materials

An apple (*Malus x domestica*) cultivar, Sekai-ichi (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.

1.2.2 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*^{2}*S*^{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).

1.2.3 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.

1.2.4 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 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.

1.2.5 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 McClue et al. (1990).

1.2.6 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 (CTCAAGCHTTGTATCATGCATAC), respectively. Flanking sequences of the 66L6-derived gene, *MdSFBB*$^{3\text{-}\alpha}$, 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 (AGAAGGATACAAGTGGAGGATG) 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 Table 1.1.

1.2.7 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).

1.2.8 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 Table 1.2. 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 "Sekai-ichi" 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.

1.2.9 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.

1.2.10 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.

1.3 Results

1.3.1 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 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 Fig. 1.1a.

1.3.2 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 1.3). 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 (Fig. 1.1a). 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 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 1.4).

1.3.3 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 (Fig. 1.1b).

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*^{4}*S*^{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 1.5). 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 1.5, Figures 1.2 and 1.3, 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 (Fig. 1.4).

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. Fig. 1.5 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.

1.3.4 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 (Fig. 1.6). 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). 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 (Fig. 1.7).

1.3.5 The S^{4sm} *haplotype, a style-specific self-compatible Japanese pear mutant, lacks the* S^4*-RNase-containing region and retains* SFBB^4 *genes*

I 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 (Fig. 1.8A). 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 between 11-17Sph-L and 11-1R, is deleted in the S^{4sm} haplotype.

Subsequently, I 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 (Fig.

1.8B). 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 (Fig. 1.8C).

1.3.6 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, I analyzed *SFBB* sequences to determine whether they show nucleotide substitution patterns similar to those of known SI genes.

I 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*. Fig. 1.9 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 1.6). 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-Poir et al. 1990) was calculated for the *SFBB* genes. Forty-eight variable sites were detected (Fig. 1.11). Four regions with particularly rich variable sites were named V1–V4 (Fig. 1.10 and Fig. 1.11). 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 1.7). These values suggest that V1 and V2 are under positive selection and that the F-box region is under purifying selection.

1.4 Discussion

1.4.1 Organization of the S *locus of Maloideae*

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

1.4.2 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. 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 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, my 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 Modlibwska 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). 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.

Table 1.1 Primers used for 3'RACE of *PpSFBBs*

SFBB	Primer name	Primer sequence (5' to 3')
PpSFBB$^{4\text{-}\alpha}$, PpSFBB$^{5\text{-}\alpha}$	PpSLFLf8	gttaatttgtctctcaaactgtc
	PpSLFLf9	tgttcttttatgtgtatatacatg
PpSFBB4$^{\text{-}\beta}$, PpSFBB$^{5\text{-}\beta}$	PpSLFLf2	agctttggtttaaattgtctctc
	PpSLFLf5	tggtggtgtttcctatgtacat
PpSFBB$^{4\text{-}\gamma}$, PpSFBB$^{5\text{-}\gamma}$	PpSLFLf6	tcctgtgtatatatatacgtgtg
	PpSLFLf7	gtgtgtaattcatgtgcatgg

Table 1.2 Primers and restriction enzymes for CAPS analysis.

SFBB	Primer name (forward/reverse)	Primer sequence (5' to 3')	PCR fragment size (bp)	Restriction enzyme	Specific flagment size (bp)
MdSFBB9-α	90FBX-F	actctcgtccaccaccacttgtat	827	*Eco*NI	305, 522
	90FBX-R	caatcgttagtagttttgtcc			
MdSFBB9-β	34FBX-F	actttcatcctccactggtat	708	*Sph*I	576, 132
	34FBX-R2	taacgagagcaatagaagtgatg			
MdSFBB3-α	66FBX-F	actctcatcctccacttgtat	744	*Hin*dIII	342, 402
	66FBX-R	gaatcctcactcctatcataac			
MdSFBB3-β	66FBX-F	actctcatcctccacttgtat	741	*Pvu*II	461, 280
	72FBX-R	gaatcctcacttggattatagg			
PpSFBB4-α	PpSLFLf9-b4n1	tgttcttttatgtgtatatacatg	1393	*Kpn*I	1153, 240
	SLFLr12-kou1GSP1	cactttcaaatagaataacattac			
PpSFBB4-β	PpSLFLf5-3RACEn3	tggtggtgtttcctatgtacat	1341	*Dra*I	1341
	SLFLr13-kou2	catacaaattaaatagaagaaaatg			
PpSFBB4-γ	PpSLFLf7-kikn1	gtgtgtaattcatgtgcatgg	967	*Nsp*I	226, 741
	SLFLr11-kou3GSPr1	ggaaggctdttcgtaactac			
PpSFBB5-α	PpSLFLf9-b4n1	tgttcttttatgtgtatatacatg	393	*Hga*I	109, 284
	PpSLFLr5-RTkou1	gtaaagctgtacattgtcttgaa			
PpSFBB5-β	PpSLFLf5-3RACEn3	tggtggtgtttcctatgtacat	1347	*Eco*NI	536, 811
	SLFLr13-kou2	catacaaattaaatagaagaaaatg			
PpSFBB5-γ	PpSLFLf7-kikn1	gtgtgtaattcatgtgcatgg	967	*Afl*II	547, 420
	SLFLr11-kou3GSPr1	ggaaggctdttcgtaactac			

Table 1.3 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
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 ($4e^{-88}$)

Table 1.4 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 1.5 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 1.4 legend for other abbreviations.

Table 1.6 *Ka/Ks*-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

Table 1.7 *Ka/Ks*-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

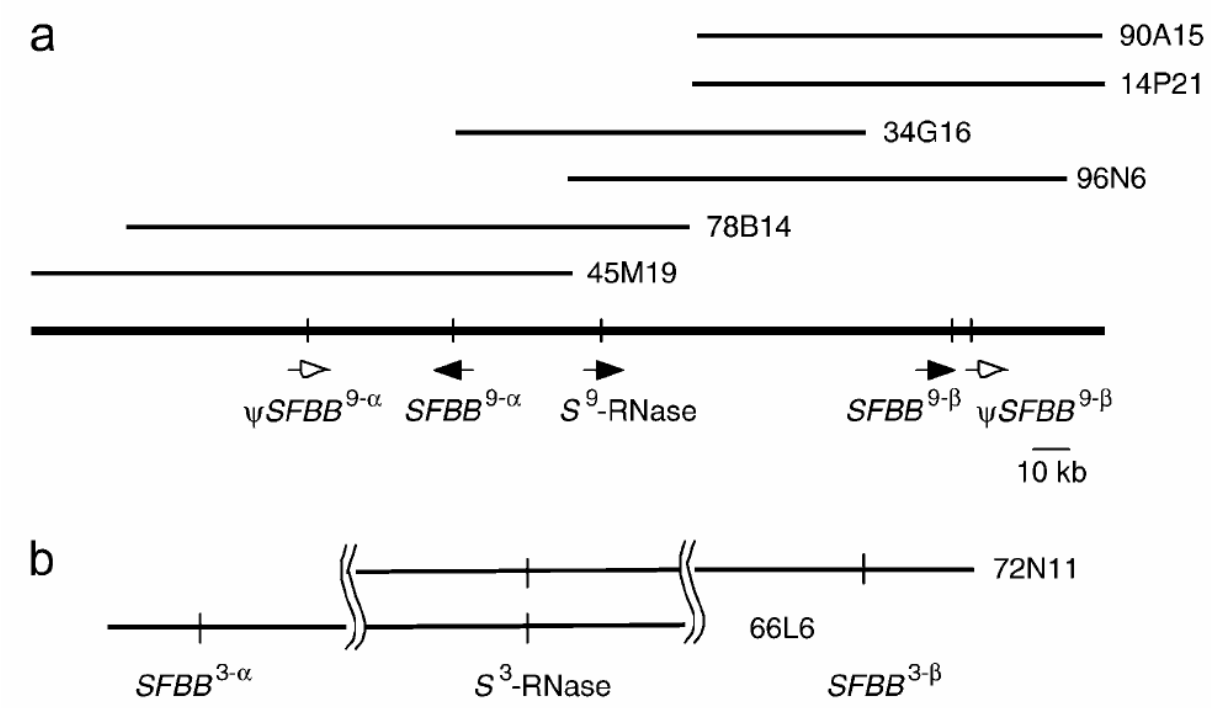

Fig.1.1　The *S* locus BAC contig of apple.

(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.

Fig. 1.2 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 1.4 and 1.5. Conserved sites and relatively conservative sites are marked with asterisks and dots, respectively. Double-headed arrow shows the F-box region.

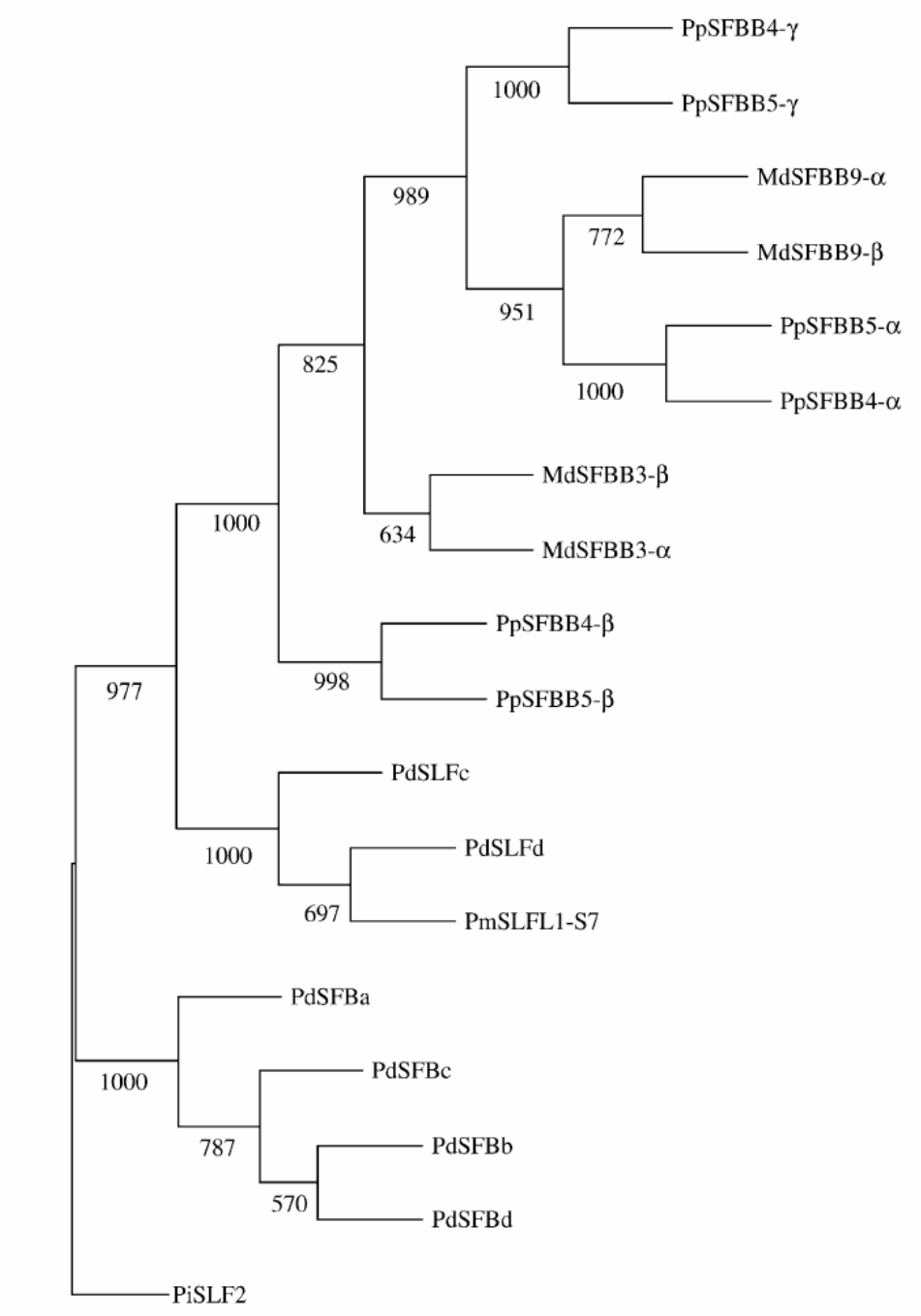

Fig. 1.3　Neighbor-joining tree of the *S* locus F-box genes with 1000 bootstraps.

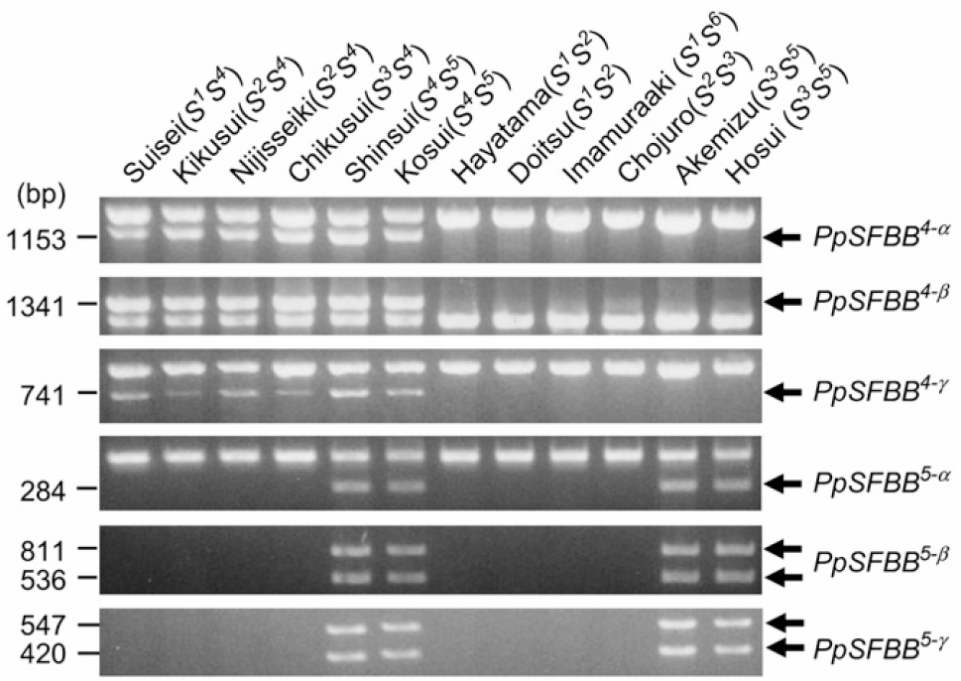

Fig. 1.4　*S*-haplotype-specific sequence polymorphism of Japanese pear *SFBB* genes. Japanese pear cultivars with different *S* genotypes were subjected to CAPS analysis for *SFBB* genes.

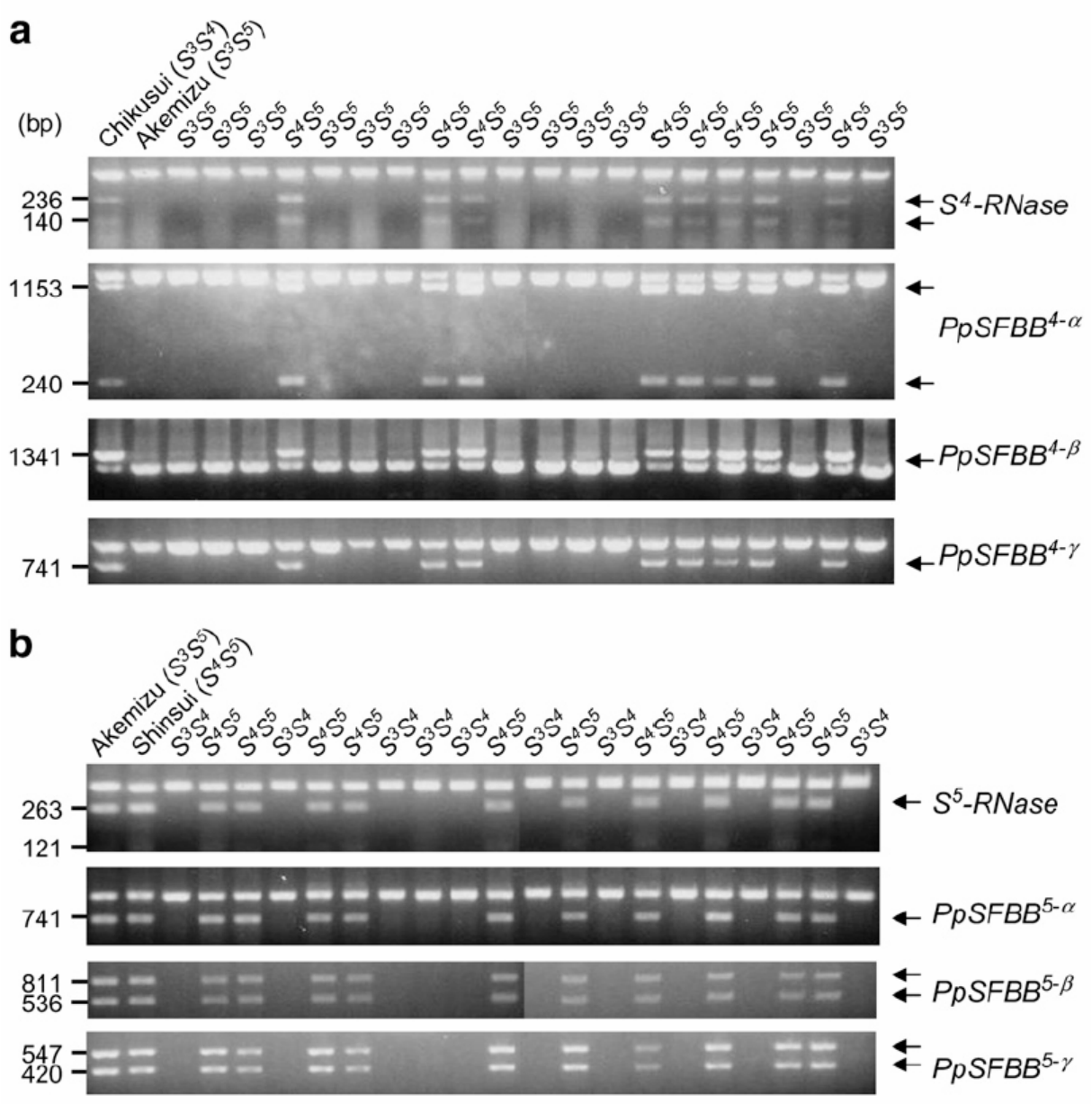

Fig. 1.5　Linkage analysis of *SFBB* genes and the *S-RNase* genes in Japanese pear.

(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.

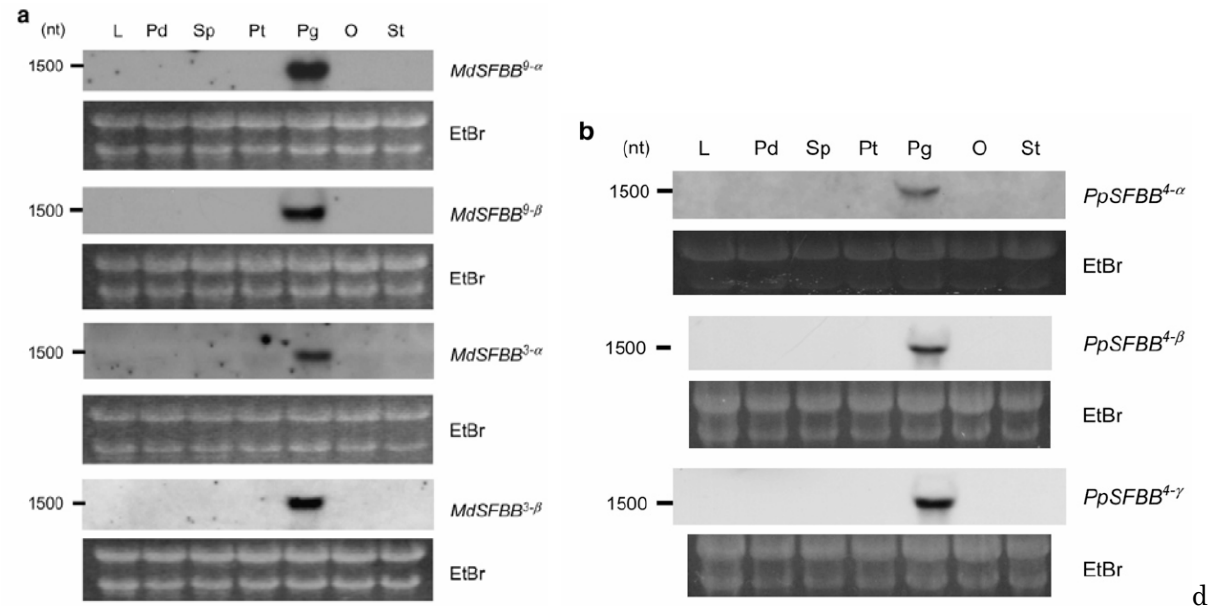

Fig. 1.6　RNA gel blot analysis of *SFBB* genes.

(a) Apple *SFBB* genes. (b) Japanese pear *SFBB* genes. Lf, leaf; Pd, pedicel; Sp, sepal; Pt, petal; Pg, pollen grain; Ov, ovary; St, style.

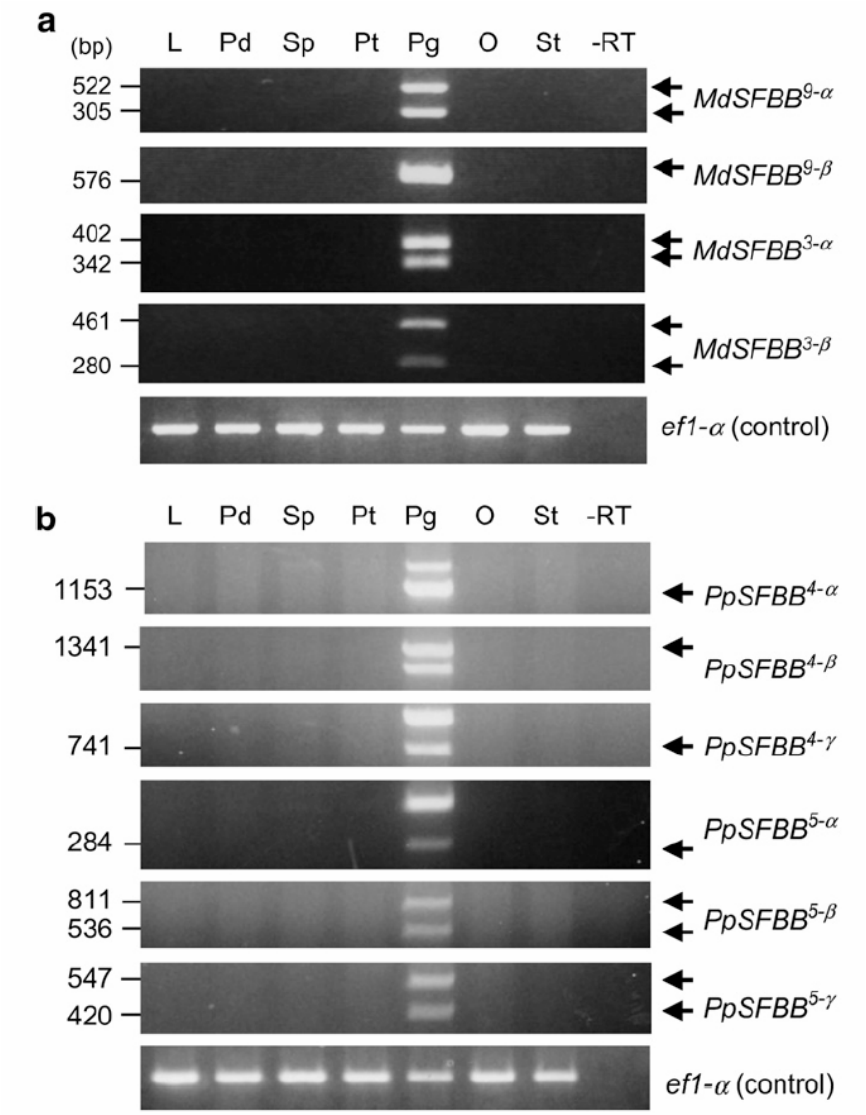

Fig. 1.7　RT–PCR/CAPS analysis of *SFBB* expression in different organs.

(a) Apple *SFBB* genes. (b) Japanese pear *SFBB* genes. –RT, pollen grain RNA negative control experiment performed without reverse transcriptase.

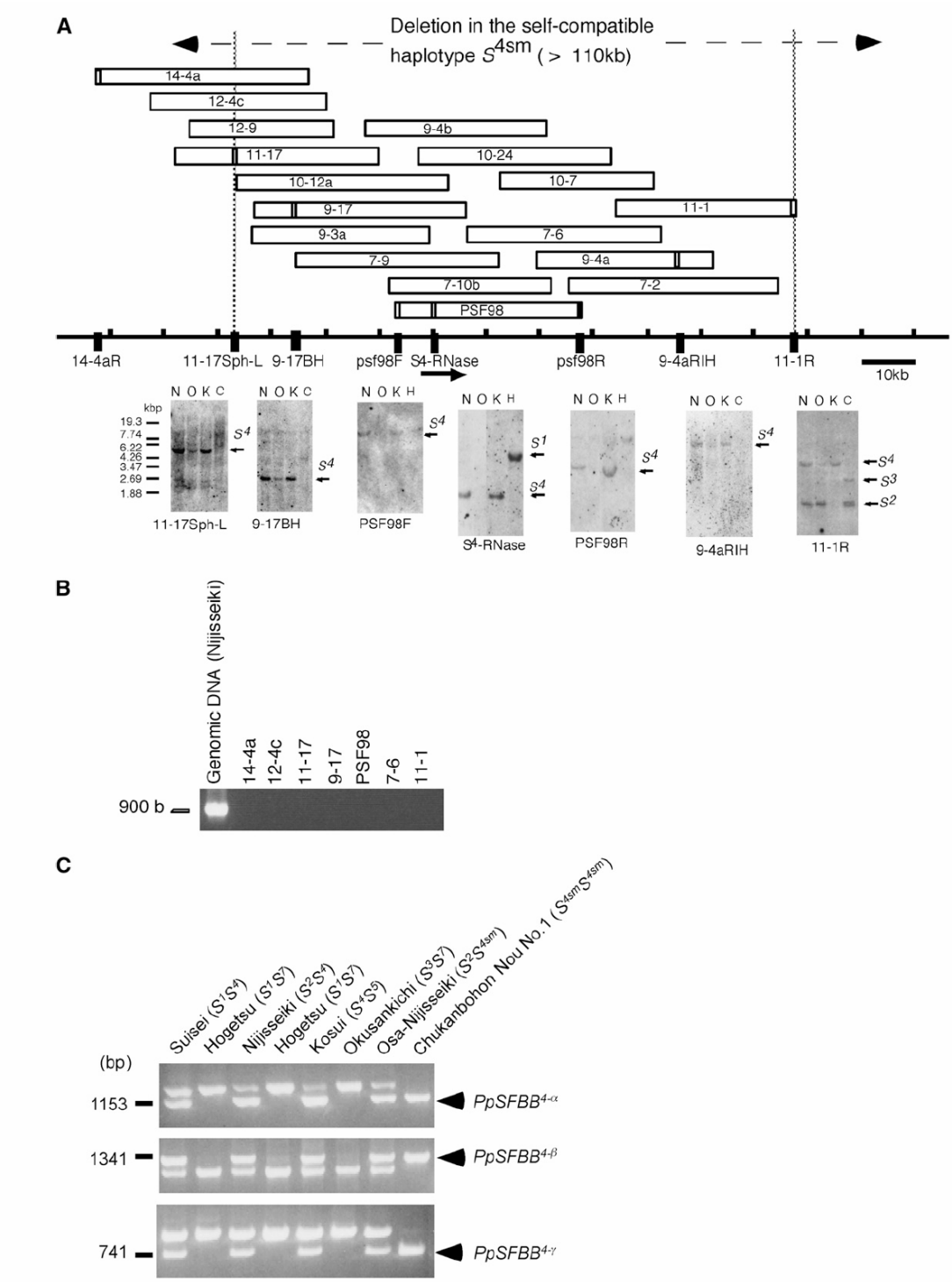

Fig. 1.8　Analysis of the style-specific, self-compatible haplotype S^{4sm}.

(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* genotype

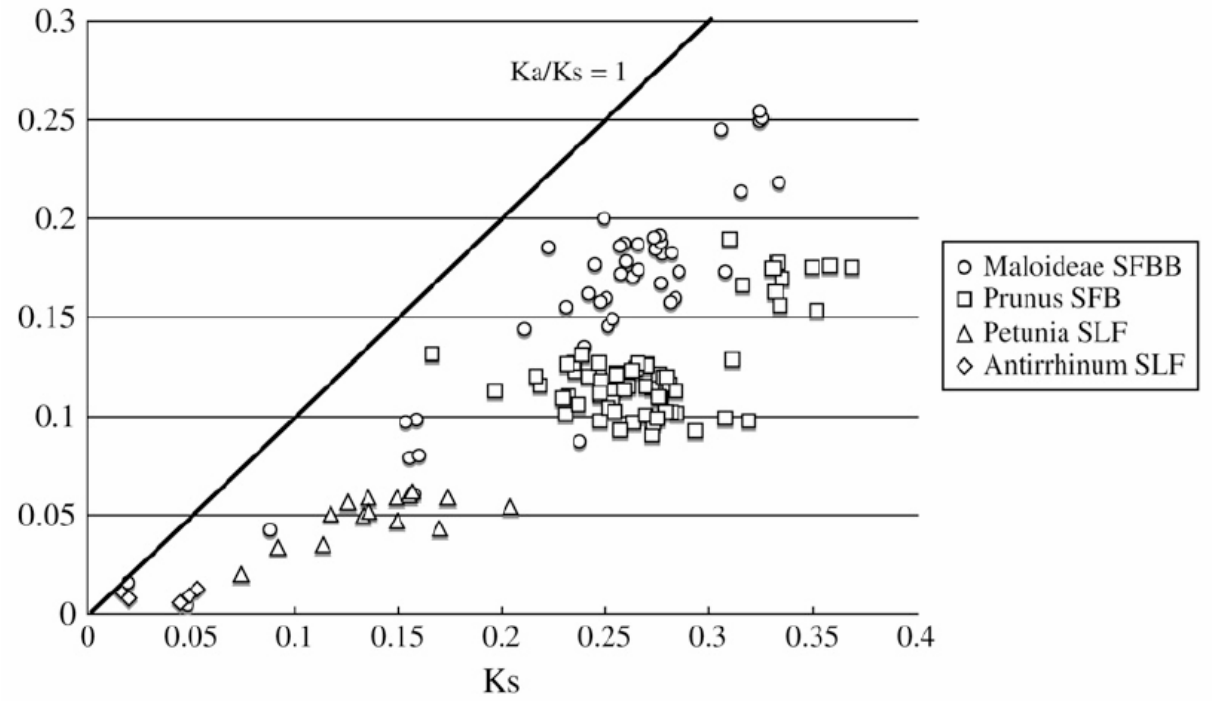

Fig. 1.9　Pairwise comparisons of synonymous (K_s) and nonsynonymous (K_a) substitution frequencies in the *SFBB* and other *S* locus F-box genes.

Circles, squares, triangles, and diamonds denote the data for *SFBB* of Maloideae, *SFB* of *Prunus*, *SLF* of *Petunia*, and *SLF* of *Antirrhinum*, respectively.

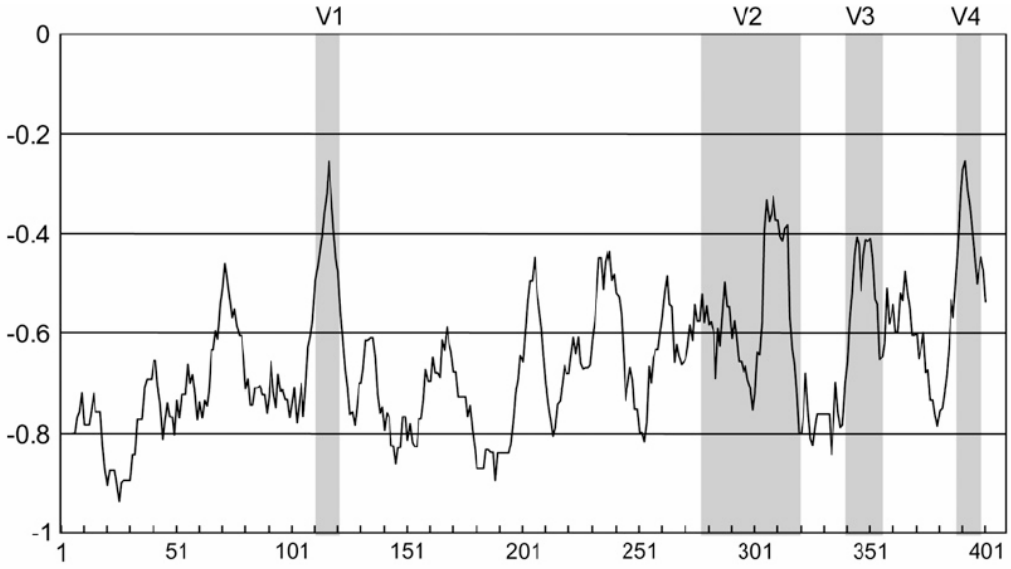

Fig. 1.10　Window-averaged plot of normed variability index at each site in the alignment of the *SFBB* genes.

Variable regions are shaded.

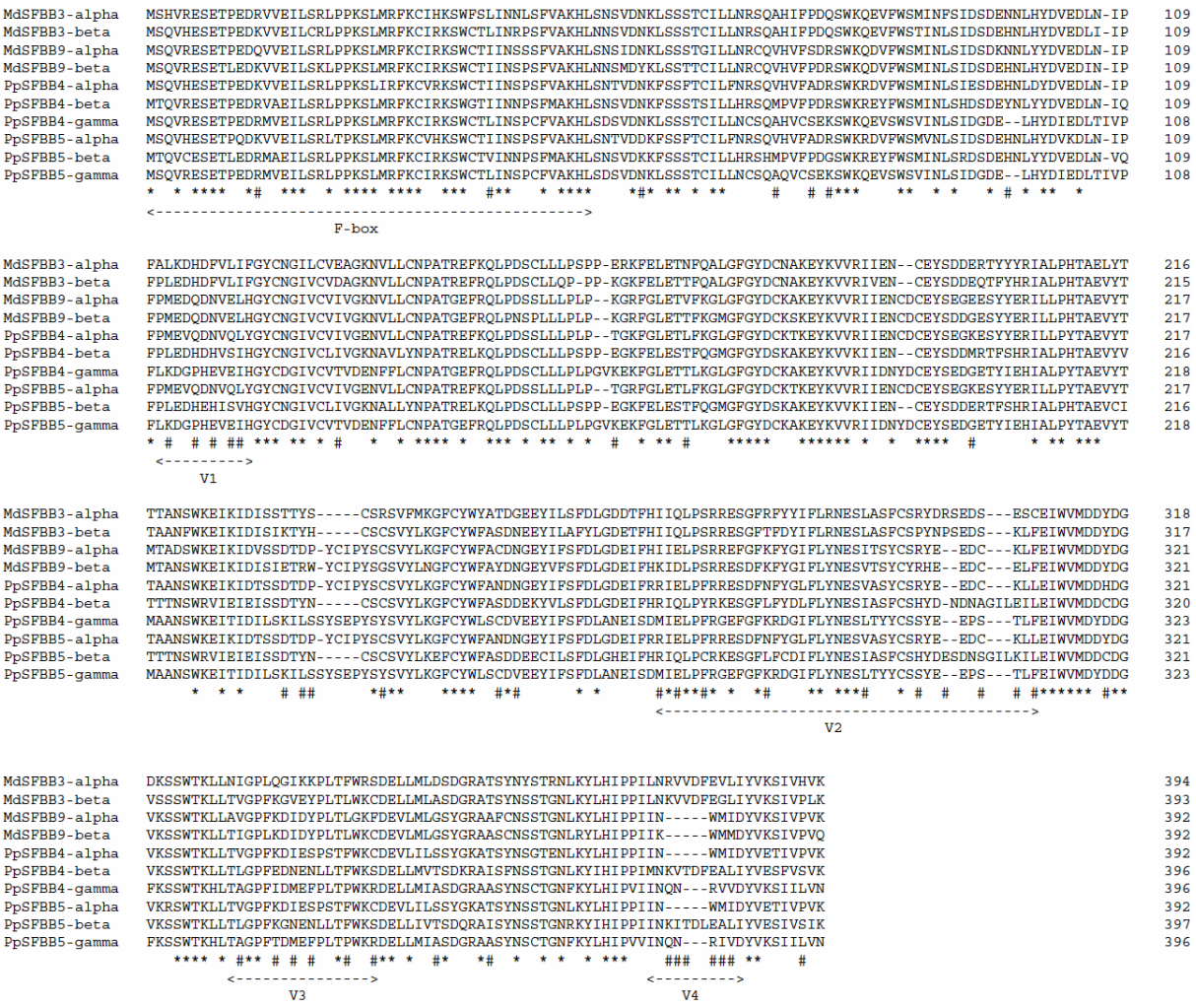

Fig. 1.11 Amino acid sequence alignment of SFBBs for NVI analysis.

Conserved sites and most variable sites with NVI values of >-0.25 were marked with asterisks and #, respectively. Double-headed arrows denote F-box and variable regions.

Chapter 2

Analysis of Japanese pear *SFBB* and its use for *S*-genotyping

2.1 Introduction

Self-incompatibility (SI) in flowering plants is a genetic system that prevents self-fertilization by enabling the pistil to reject pollen from genetically related individuals, thus promoting outcrossing (de Nettancourt 2001). The specificity of the SI response is determined by the haplotyes of the polymorphic *S* locus, which contains at least one gene for the pistil determinant (pistil *S*) and one for the pollen determinant (pollen *S*).

The families Rosaceae, Solanaceae and Plantaginaceae include species exhibiting S-RNase-based gametophytic self-incompatibility (GSI) in which the pistil *S* gene encodes a polymorphic S-RNase (Kao and Tsukamoto 2004). *S-RNase* has been characterized in detail, while the nature of pollen *S* gene had been unknown for a long time. Recently, *SLF* (*S* locus F-box gene) and *SFB* (*S* haplotype specific F-box gene) were identified as (putative) pollen *S* genes in the three families (McClure 2004). The identity of *SLF* as the pollen *S* determinant of *Petunia inflata*, a solanaceous species, was demonstrated by transformation experiment (Sijacic et al. 2004). In several *Prunus* species of the Rosaceae, analysis of pollen-part self-compatible mutants revealed insertion/deletion or complete loss of *SFB* gene, suggesting that *SFB* is the pollen *S* (Ushijima et al. 2004; Sonneveld et al. 2005; Vilanova et al. 2006).

The family Rosaceae is divided into four subfamilies; Spiraeoideae, Rosoideae, Amygdaloideae and Maloideae (Morgan et al. 1994). In Maloideae, that includes apple (*Malus × domestica*) and Japanese pear (*Pyrus pyrifolia*), *S-RNase* has been studied extensively (Sassa et al. 1992, 1996; Ishimizu et al. 1996, 1999; Ushijima et al. 1998; Castillo et al. 2002; Takasaki et al. 2004), and pollen *S* gene candidates, *SFBB* (S locus F-box brothers) genes, were identified most recently (Sassa et al. 2007). *MdSFBB*$^{9-\alpha}$ and *MdSFBB*$^{9-\beta}$ were identified from a sequenced apple *S*^{9} haplotype region of 317 kb. *MdSFBB*$^{3-\alpha}$ and *MdSFBB*$^{3-\beta}$

were isolated from apple *S*[3] haplotype BAC clones, and each three *PpSFBB* genes were isolated from Japanese pear *S*[4] and *S*[5] haplotypes. Sequence comparisons of *SFBB* genes revealed that apple *MdSFBB* genes were more homologous to that from same haplotype than that from different haplotype (i.e., amino acid sequence homology between *MdSFBB*$^{3-\alpha}$ and *MdSFBB*$^{3-\beta}$ is 82.2%, while *MdSFBB*$^{3-\alpha}$ and *MdSFBB*$^{9-\alpha}$ is 70.5%), while Japanese pear *PpSFBB* genes showed high homologies within the same groups from different haplotypes (i.e., amino acid sequence homology between *PpSFBB*$^{4-\alpha}$ and *PpSFBB*$^{5-\alpha}$ is 96.4%, while *PpSFBB*$^{4-\alpha}$ and *PpSFBB*$^{4-\beta}$ is 66.9%). These *SFBB* genes exhibited pollen-specific expression, *S* haplotype sequence specificity and linkage to the *S* locus, supporting the idea that they are the pollen *S* genes. Pollen-expressed F-box genes, named *MdSLF1* and *MdSLF2*, were also isolated from apple *S*[1] and *S*[2] haplotypes as pollen *S* candidates (Cheng et al. 2006). *MdSLF* share high homology with *MdSFBB* (67.5–75.4%), however, it is not clear at present whether *S*[1] and *S*[2] haplotypes contain other related F-box genes. Although the features of the *SFBB* genes suggest that they are good pollen *S* candidates, whether all of the multiple *SFBB*s in a haplotype are involved in pollen *S* specificity is not clear, and the possibility that none of them are pollen determinant can not be excluded at present. Among the *PpSFBB* groups of Japanese pear, *PpSFBB*$^{-\gamma}$ would be the most problematic group for the pollen determinant because it has the highest amino acid identity between *S*[4] and *S*[5] haplotypes (99.0%, Sassa et al. 2007).

Because of their SI, most Japanese pear cultivars need cross-pollination for stable fruit set. Determination of the *S* genotypes of cultivars is thus important for selection of appropriate pollen donors in the fruit production. To date, several *S-RNase*-based *S* genotyping systems have been reported (Ishimizu et al. 1999; Castillo et al. 2002; Takasaki et al. 2004; Kim et al. 2007). These systems identify *S* genotypes by CAPS analysis; amplification of the all *S-RNase*s by degenerate primer pairs followed by digestion with allele-specific restriction enzymes. However, because of the high level of sequence diversity of the *S-RNase* genes, the discovery of new *S* haplotype required re-design of new degenerate primers to amplify the

new *S-RNase* gene for *S* genotyping (Takasaki et al. 2004; Kim et al. 2007). *S* locus gene that is less diverged than the *S-RNase* gene can be more feasible for the use in CAPS-based *S* genotyping system.

I 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 more than 110 kb region containing the S^4-*RNase* gene (Sassa et al. 1997, 2007). To date, the S^{4sm} haplotype is known as the only mutation that confers self-compatibility in Japanese pear, and thus is an important resource for the breeding of self-compatible cultivars. DNA marker-assisted selection should facilitate the breeding of self-compatible cultivars, however, by using the *S-RNase*-based *S* genotyping system, it is difficult to identify self-compatible lines in a segregating population because the S^{4sm} haplotype is the deletion mutant of the *S-RNase* gene.

In this article, I analyzed *PpSFBB*$^{-Y}$ genes which exhibit lowest sequence diversity between S^4 and S^5 haplotypes. I isolated nine *PpSFBB*$^{-Y}$ genes from different *S* haplotypes and analyzed their sequence polymorphisms. Based on the polymorphisms, I developed a new *S* genotyping system using *PpSFBB*$^{-Y}$ genes. The system was found to be useful not only for typing the known S^1–S^9, but also for identifying the non functional haplotype S^{4sm} and the new *S* haplotypes such as S^k.

2.2 Materials and methods

2.2.1 Plant materials

Thirteen Japanese pear (*P. pyrifolia*) cultivars were used; Hayatama (S^1S^2), Suisei (S^1S^4), Imamura-aki (S^1S^6), Ichiharawase (S^1S^8), Chojuro (S^2S^3), Nijisseiki (S^2S^4), Hosui (S^3S^5), Niitaka (S^3S^9), Kosui (S^4S^5), Shinkou (S^4S^9), Okusankichi (S^5S^7), Chukanbohon Nou No.1 ($S^{4sm}S^{4sm}$) and Kinchaku (S^4S^k).

2.2.2 Cloning and sequence analysis of PpSFBB^{-Y}

DNA was extracted from young leaves as previously described (Sassa 2007) and used for PCR. *PpSFBB*$^{-Y}$ genes were amplified by using PpFBXgf7 (5′-gtgtgtaattcatgtgcatgg-3′) and PpFBXgr3 (5′-tggaacgtttcccctcaactc-3′) primers, which were designed to amplify full length of *PpSFBB*$^{-Y}$ coding region. Standard PCR conditions (in 20 µl total volume) included 40 ng of genomic DNA, 0.4 µM of each primer, 200 µM dNTP, 1× PCR Buffer, 1 U of ExTaq (TaKaRa). PCR amplification was carried out for 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 1.5 min, and final extension at 72°C for 3 min. The PCR products were cloned into a plasmid vector and sequenced. Sequence data was analyzed with BioEdit ver.7.0 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and ClustalW (Thompson et al. 1994).

2.2.3 CAPS and dCAPS analysis

PpSFBB^{-Y} fragments were amplified by using PpFBXf7 and PpFBXr3 were subjected to CAPS (cleaved amplified polymorphic sequence) analysis. PCR products were digested with *Taq*I, *Bbv*CI, *Nsp*I, *Afl*II, *Dde*I, *Psi*I, *Sma*I, *Hae*III or *Apo*I. *Taq*I and *Sma*I were reacted for 3 h at 65 and 30°C, respectively, and the other endonucleases were incubated for 3 h at 37°C. Expected fragment sizes are shown in Table 2.1. These products were separated on 1% (target fragment >1 kbp) or 2% agarose gels (<1 kbp) and were visualized by staining with ethidium bromide.

For the detection of *PpSFBB*$^{2-Y}$, derived cleaved amplified polymorphic sequence (dCAPS) analysis was performed (Neff et al. 1998). *PpSFBB*$^{-Y}$ fragments were amplified with 1 bp mutated primer GdCAPSS2g1-Rsa (5′-taaaatattatcatcatatagcgaacGta-3′) and reverse primer PpFBXr11 (5′-ggaaggctcttcgtaactac-3′). PCR amplification was carried out for 30 cycles of denaturation at 98°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C 3 min. PCR products were digested with *Rsa*I for 3 h at 37°C and separated by 3% agarose gel. CAPS analysis of the *S-RNase* genes was performed according to pervious

report (Takasaki et al. 2004).

2.2.4 Accession numbers

The DDBJ/EMBL/GenBank accession numbers for the sequences shown in Fig. 2.1 are AB297933 (*PpSFBB*$^{1-Y}$), AB297934 (*PpSFBB*$^{2-Y}$), AB297935 (*PpSFBB*$^{3-Y}$), AB297936 (*PpSFBB*$^{6-Y}$), AB297937 (*PpSFBB*$^{7-Y}$), AB297938 (*PpSFBB*$^{8-Y}$), AB297939 (*PpSFBB*$^{9-Y}$) and AB297940 (*PpSFBB*$^{k-Y}$).

2.3 Results

2.3.1 Cloning and sequence comparison of PpSFBB^{-Y} *genes*

PpSFBB^{-Y} genes from different cultivars were amplified by PCR; 'Suisei' (S^1 S^4), 'Nijisseiki' (S^2 S^4), 'Hosui' (S^3 S^5), 'Imamura-aki' (S^1 S^6), 'Okusankichi' (S^5 S^7), 'Ichiharawase' (S^1 S^8) and 'Niitaka' (S^3 S^9). Together with *PpSFBB*$^{4-Y}$, a new sequence was isolated from 'Suisei' (S^1 S^4), and was named *PpSFBB*$^{1-Y}$. Similarly, new sequences obtained from 'Nijisseiki' (S^2 S^4) and 'Hosui' (S^3 S^5) were designated as *PpSFBB*$^{2-Y}$ and *PpSFBB*$^{3-Y}$, respectively. In addition to the S^1–S^5 sequences of the *PpSFBB*$^{-Y}$ gene, *PpSFBB*$^{6-Y}$, *PpSFBB*$^{7-Y}$, *PpSFBB*$^{8-Y}$ and *PpSFBB*$^{9-Y}$ were amplified from 'Imamura-aki' (S^1 S^6), 'Okusankichi' (S^5 S^7), 'Ichiharawase' (S^1 S^8) and 'Niitaka' (S^3 S^9), respectively. In all cases the 1,245 bp cloned fragment contained the full length (1,191 bp) of *PpSFBB*$^{-Y}$ coding regions. Amino acid sequences encoded by these genes showed very high sequence homology with those of *PpSFBB*$^{4-Y}$ and *PpSFBB*$^{5-Y}$ (Fig. 2.1, Table 2.2).

2.3.2 Development of a new S *genotyping system using* PpSFBB^{-Y} *genes*

Based on the sequence polymorphisms of *PpSFBB*$^{-Y}$, a new CAPS/dCAPS system was established for *S* genotype assignments in Japanese pear harboring S^1–S^9 haplotypes. *PpSFBB*$^{-Y}$ fragments were amplified from 11 cultivars, 'Hayatama' (S^1 S^2), 'Suisei' (S^1 S^4),

'Imamura-aki' ($S^1 S^6$), 'Ichiharawase' ($S^1 S^8$), 'Chojuro' ($S^2 S^3$), 'Nijisseiki' ($S^2 S^4$), 'Hosui' ($S^3 S^5$), 'Niitaka' ($S^3 S^9$), 'Kosui' ($S^4 S^5$), 'Shinkou' ($S^4 S^9$) and 'Okusankichi' ($S^5 S^7$) using the specific primers, PpFBXf7 and PpFBXr3. Based on the nine *PpSFBB*$^{-Y}$ sequences, I selected restriction endonucleases which digest specific *PpSFBB*$^{-Y}$ fragments; S^1–*Taq*I, S^3 and S^5-*Bbv*CI, S^4-*Nsp*I, S^5-*Afl*II, S^6-*Dde*I, S^7-*Psi*I, S^8-*Sma*I and S^9-*Hae*III. Although *Bbv*CI digestion detects both S^3 and S^5 haplotypes, these two haplotypes can be distinguished by *Afl*II that specifically digests the S^5 haplotype. Estimated restriction fragment sizes are shown in Table 2.1. The result of the CAPS analysis showed that the observed band sizes were consistent with the expected ones (Fig. 2.2). *PpSFBB*$^{2-Y}$ specific fragment was detected by dCAPS system (Neff et al. 1998). First, *PpSFBB*$^{-Y}$ genes were amplified with 1 bp mutated primer GdCAPSS2f1-Rsa and PpFBXr11 (Fig. 2.3). The amplified fragments (232 bp) were digested with *Rsa*I and analyzed on a 3% agarose gel. *PpSFBB*$^{2-Y}$ specific fragment was detected at 156 bp by primer-induced *Rsa*I site (Figs. 2.2b, 2.3). Taken together, the CAPS and dCAPS analyses of *PpSFBB* represent a new *S* genotyping system.

2.3.3 Detection of PpSFBB^{-Y} *genes from the stylar part self-compatible mutant and* S^k *haplotype*

The S^{4sm} haplotype has been discovered in a self-compatible cultivar 'Osa-Nijisseiki'. Sassa et al. (1997, 2007) have shown that the S^{4sm} haplotype lacks more than 110 kb region containing the *S*^{4}*-RNase*. Ishimizu et al. (1999) showed that the S^{4sm} haplotype was not detectable in 'Akibae' ($S^{4sm} S^5$) using the *S-RNase*-based *S* genotyping system. Aiming to identify the S^{4sm} haplotype, I performed the *S-RNase* and *PpSFBB*$^{-Y}$-based *S* genotyping of 'Chukanbohon Nou No.1' ($S^{4sm} S^{4sm}$). Fig. 2.4 shows detection of *PpSFBB*$^{4-Y}$ and S^4*-RNase* using the CAPS systems. A *PpSFBB*$^{4-Y}$ specific fragment was detected in 'Chukanbohon Nou No.1' (lane 3, Fig. 2.4a), while *S*^{4}*-RNase* was not (lane 3, Fig. 2.4b). This result suggests that the S^{4sm} haplotype can be detected by combining of the *S-RNase*- and *PpSFBB* $^{-Y}$-based *S* genotyping systems; the presence of *PpSFBB* $^{4-Y}$ but S^4*-RNase* indicates the S^{4sm} haplotype.

In a previous study (Kim et al. 2007), the *S* genotype of Japanese pear cultivar 'Kinchaku' was found to be $S^4 S^k$, and a new S^k -*RNase* was cloned (Kim et al. 2007). However, this new *S-RNase* gene was not amplified by the previously reported primers for *S* genotyping, FTQQYQ and anti-(I/T) IWPNV (Takasaki et al. 2004; Kim et al. 2007), and a new primer was designed to detect the S^k haplotype in addition to S^1–S^9 (Kim et al. 2007). To test whether *PpSFBB*$^{-\gamma}$ can be amplified from the newly identified S^k haplotype, PCR was performed using PpFBXgf7 and PpFBXgr3 as a primer pair, and digested with *Nsp*I that cleaves *PpSFBB*$^{4-\gamma}$ specifically. Undigested PCR product from 'Kinchaku' was detected, indicating that the PCR product is *PpSFBB*$^{k-\gamma}$ (Fig. 2.4a). I cloned and sequenced *PpSFBB*$^{k-\gamma}$, and found that the S^k haplotype can be detected by *Apo*I digestion of *PpSFBB*$^{-\gamma}$ (Fig. 2.4c). It was also confirmed that *PpSFBB*$^{k-\gamma}$ sequence does not generate a fragment that is similar in size to those obtained when S^1–S^9 are digested with their respective *S*-specific enzymes (data not shown), suggesting that the CAPS system is capable of detecting S^k in addition to S^1–S^9 haplotypes.

2.4 Discussion

2.4.1 Sequence polymorphism of PpSFBB$^{-\gamma}$

Previously, I isolated six F-box genes (*PpSFBB*$^{4-\alpha}$, *PpSFBB*$^{4-\beta}$, *PpSFBB*$^{4-\gamma}$, *PpSFBB*$^{5-\alpha}$, *PpSFBB*$^{5-\beta}$ and *PpSFBB*$^{5-\gamma}$) from Japanese pear cultivar 'Kosui' (S^4 S^5). These genes exhibited pollen-specific expression, linkage to the *S-RNase* and *S* haplotype-specific sequence polymorphisms (Sassa et al. 2007). Although the features of *SFBB* genes suggest that all *PpSFBB* genes are good pollen *S* candidates, whether all of the multiple *SFBB*s in a haplotype are involved in pollen *S* specificity is not clear, and the possibility that none of them are pollen determinant can not be excluded at present. In this article, I isolated *PpSFBB*$^{-\gamma}$ sequences from different *S* haplotypes and investigated their *S* haplotype sequence specificity in Japanese pear. In addition to the reported *PpSFBB*$^{4-\gamma}$ and *PpSFBB*$^{5-\gamma}$, new seven *PpSFBB*$^{-\gamma}$ sequences were isolated from S^1 to S^9 haplotypes, and were named *PpSFBB*$^{1-\gamma}$ to *PpSFBB*$^{9-\gamma}$.

Comparison of amino acid sequences of the nine *PpSFBB*$^{-\gamma}$s revealed that they showed *S* haplotype-specific polymorphisms, however, they are highly homologous with each other (97.5–99.7%). Some polymorphic sites were found outside the variable regions that were defined in a previous study (Sassa et al. 2007). Considering that for solanaceous species it was reported that hypervariable regions alone are not sufficient for *S* recognition (Kao and McCubbin 1996; Zurek et al. 1997; Verica et al. 1998), the polymorphisms found outside the variable regions of *PpSFBB*$^{-\gamma}$ may also have some effect on the *S* specificity. Therefore, the possibility that *PpSFBB*$^{-\gamma}$ genes are involved in pollen *S* specificity cannot be excluded, and should be examined by functional analysis.

2.4.2 New S *genotyping system using* PpSFBB$^{-\gamma}$

In this study, it was demonstrated that *PpSFBB*$^{-\gamma}$-based *S* genotyping system is useful for detection of S^1–S^9 haplotypes in Japanese pear. Although the *S-RNase*-based *S* genotyping systems have been reported (Ishimizu et al. 1999; Castillo et al. 2002; Takasaki et al. 2004; Kim et al. 2007), the use of CAPS/dCAPS systems for both *PpSFBB*$^{-\gamma}$ and *S-RNase* genes would lead to a more reliable determination of *S* genotypes. The use of both systems is also effective for determination of the S^{4sm} haplotype which is a deletion mutant of the *S-RNase* gene conferring stylar-part self-compatibility (Sassa et al. 1997).

Because of the high level of sequence diversity of the *S-RNase* genes, identification of new alleles has accompanied revision of the *S-RNase*-based *S* genotyping systems. For example, Takasaki et al. (2004) designed a new primer (anti-(I/T) IWPNV) to detect a newly identified *S-RNase* which was undetectable with the primers proposed by Ishimizu et al. (1999). Kim et al. (2007) replaced anti-(I/T) IWPNV with PSprI to amplify the new S^k*-RNase* from 'Kinchaku'. It is likely that further identification of new haplotypes will require revision of the primers. In contrast, the *S* genotyping system using the *PpSFBB*$^{-\gamma}$ genes would be effective for identification of new *S* haplotypes because of their high sequence homologies.

Table 2.1 Estimated restriction fragment sizes (bp) of the *PpSFBB*$^{-\gamma}$ genes

	*Taq*I	*Rsa*I	*Bbv*CI	*Nsp*I	*Afl*II	*Dde*I	*Psi*I	*Sma*I	*Hae*III	*Apo*I
S1	80/165/***430***/570	47/185	184/1,061	1,245	1,245	41/58/143/488/515	118/1,127	510/735	175/199/871	31/57/102/257/363/435
S2	80/570/595	29/47/***156***	184/1,061	1,245	1,245	58/184/488/515	118/1,127	510/735	175/199/871	31/57/102/257/363/435
S3	80/570/595	232	***1,245***	1,245	1,245	58/184/488/515	118/1,127	510/735	175/199/871	31/57/102/257/363/435
S4	52/80/543/570	232	184/1,061	224/***1,021***	1,245	58/184/488/515	118/1,127	510/735	175/199/871	31/57/102/257/363/435
S5	80/570/595	47/185	***1,245***	1,245	***544/701***	58/184/488/515	118/1,127	510/735	175/199/871	31/57/102/257/363/435
S6	80/570/595	47/185	184/1,061	1,245	1,245	41/58/96/143/***392***/515	118/1,127	510/735	175/199/871	31/57/102/257/363/435
S7	80/570/595	232	184/1,061	1,245	1,245	41/58/143/488/515	118/207/***920***	510/735	175/199/871	31/57/102/257/363/435
S8	80/570/595	47/185	184/1,061	1,245	1,245	41/58/143/488/515	118/1,127	***1,245***	175/199/871	31/57/102/257/363/435
S9	80/570/595	232	184/1,061	1,245	1,245	58/184/488/515	118/1,127	510/735	175/***1,070***	31/57/102/257/363/435
Sk	80/570/595	47/185	184/1,061	1,245	1,245	41/58/143/488/515	118/1,127	510/735	175/199/871	57/102/257/363/***466***

Representative *S* haplotype-specific fragments are indicated in bold and italics.

The *PpSFBB*$^{2\text{-}\gamma}$ specific fragments were detected by the dCAPS system (see text)

Table 2.2 Number of amino acid differences among the PpSFBB-γs

	S2	S3	S4	S5	S6	S7	S8	S9	Sk
S1	4	2	2	4	4	1	1	5	4
S2	–	4	3	1	4	4	3	6	4
S3		–	2	4	6	3	3	4	6
S4			–	4	4	3	3	5	6
S5				–	5	4	5	7	4
S6					–	5	5	10	5
S7						–	6	6	5
S8							–	6	5
S9								–	8

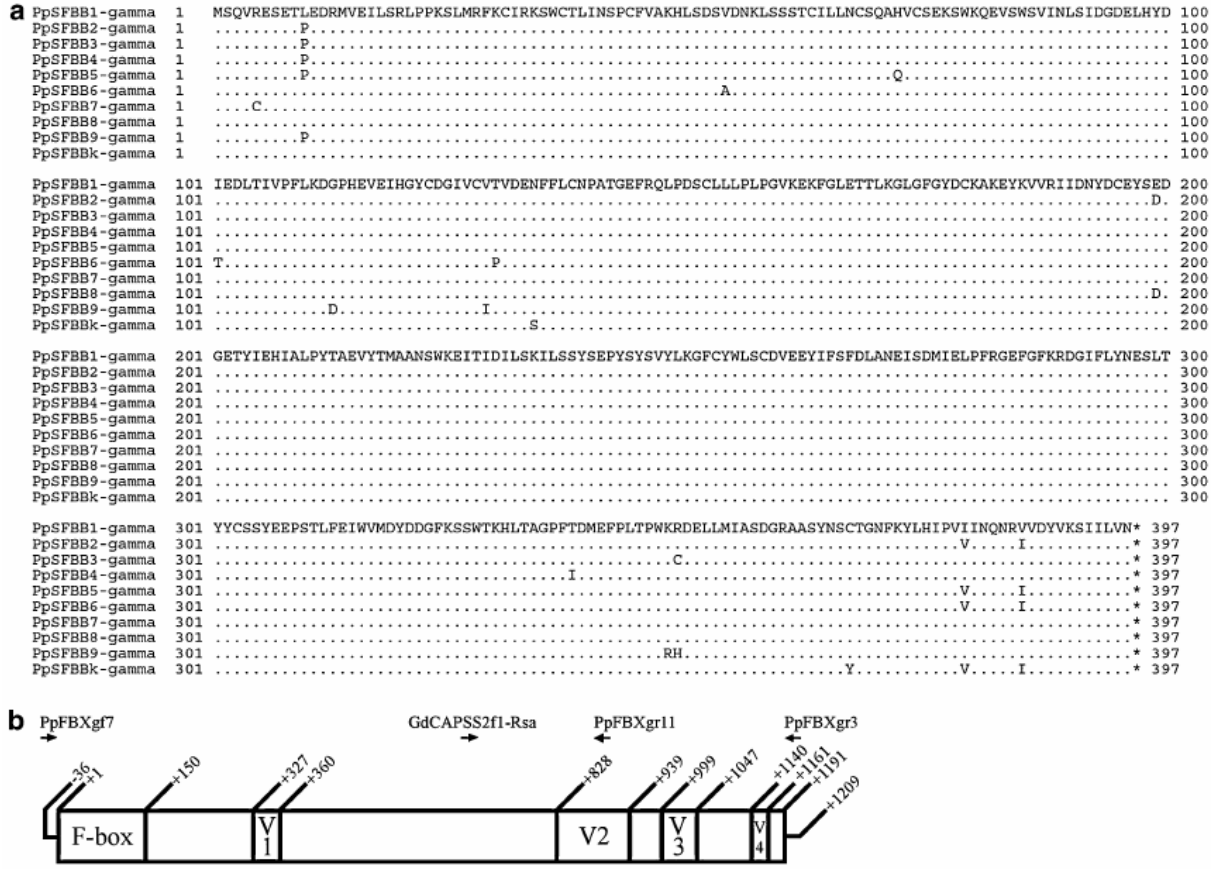

Fig. 2.1 Structure of the *PpSFBB*$^{-Y}$ genes.

(a) Amino acid sequences of PpSFBB^{-Y} were aligned by using ClustalW. *Dots* indicate identical amino acids. (b) Schematic representation of the *PpSFBB*$^{-Y}$ genes. Adenine of the start codon was positioned to be +1. F-box region and variable regions were defined in a previous study (Sassa et al. 2007). *Arrows* indicate positions and directions of primers used in this study.

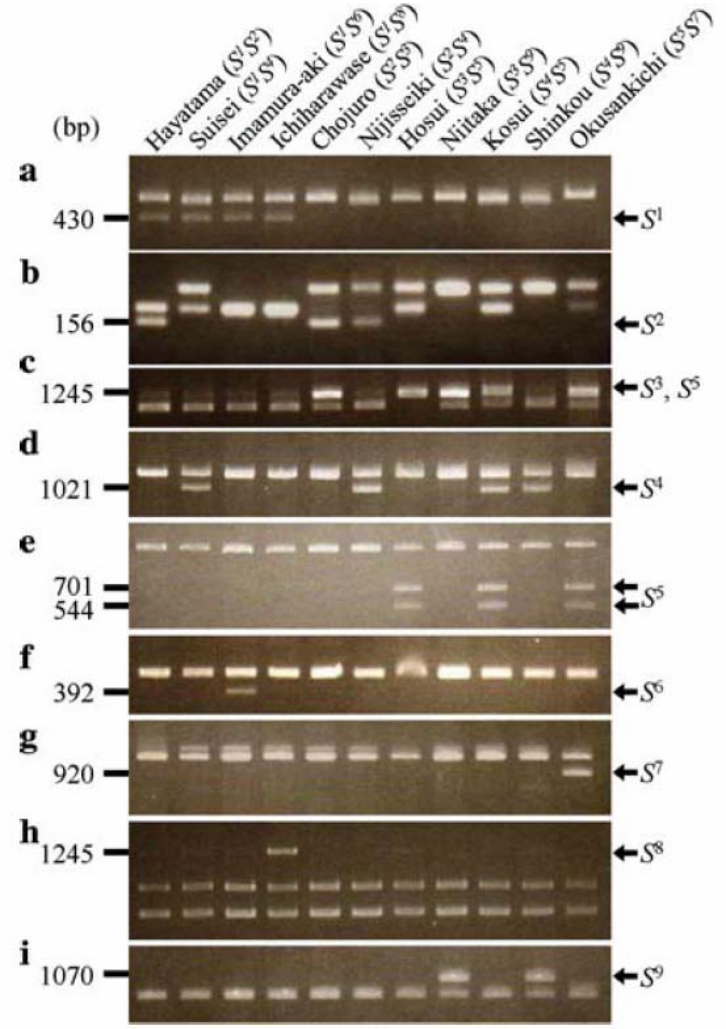

Fig. 2.2　*S* haplotype-specific sequence polymorphisms of *PpSFBB*$^{-\gamma}$ genes.

The *S* haplotype-specific fragments were detected by CAPS and dCAPS analysis. Amplified *PpSFBB*$^{-\gamma}$ genes were digested with nine restriction endonucleases; *Taq*I (a), *Rsa*I (b), *Bbv*CI (c), *Nsp*I (d), *Afl*II (e), *Dde*I (f), *Psi*I (g), *Sma*I (h) and *Hae*III (i). *Arrows and numbers* show the representative *S* haplotype specific fragments and their sizes (bp), respectively (see Table 2.1).

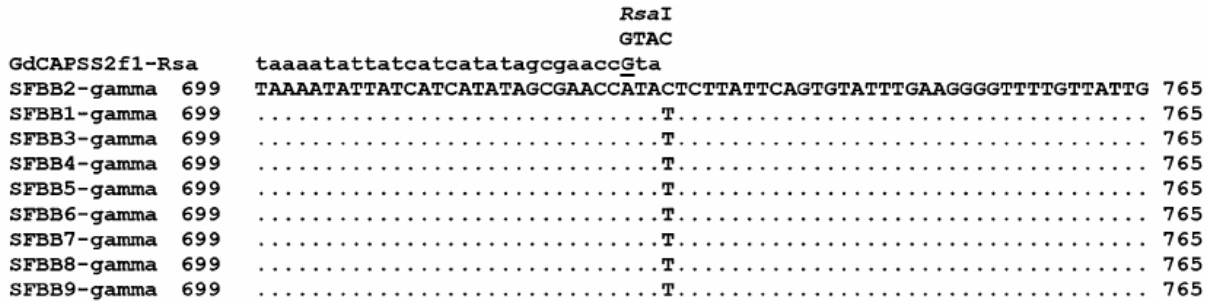

Fig. 2.3 dCAPS analysis of the *PpSFBB*2-γ.

Partial sequences of the nine *PpSFBB*-γ genes (699–765 bp) and 1 bp mutated dCAPS primer GdCAPSS2f1-Rsa are shown. Dots indicate identical nucleotides. The dCAPS primer introduces an *Rsa*I site at 728 bp of *PpSFBB*2-γ

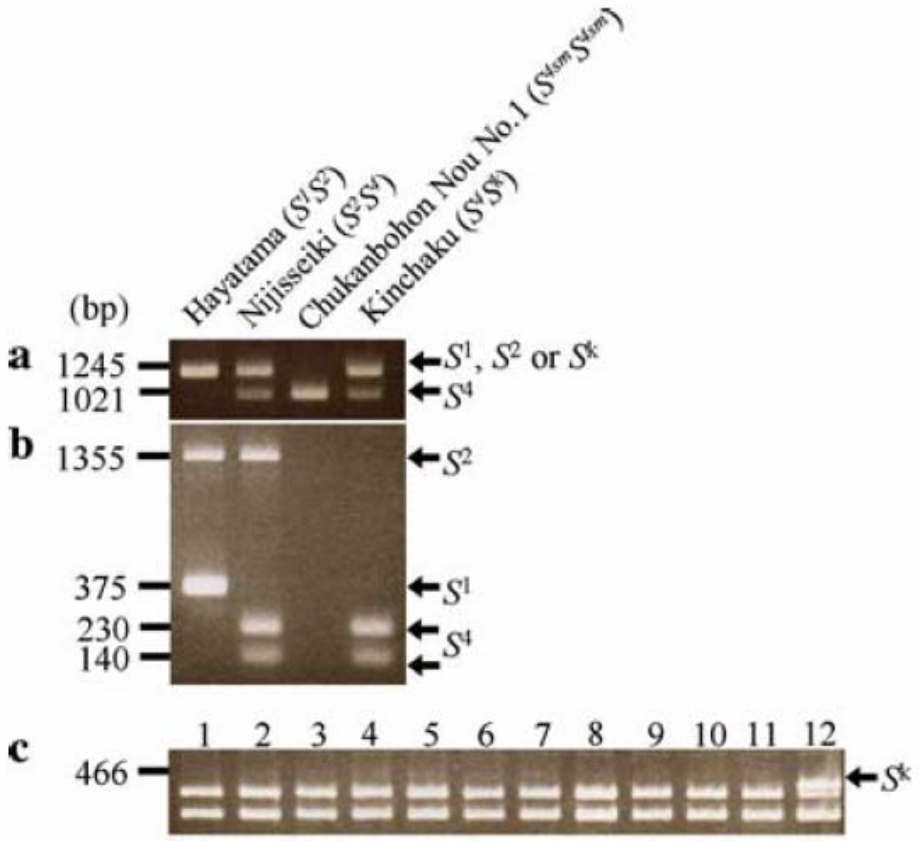

Fig. 2.4 Identification of the S^{4sm} haplotype and detection of *PpSFBB*$^{k-Y}$.

(a) CAPS analysis of the *PpSFBB*$^{4-Y}$. *PpSFBB*$^{-Y}$ fragments were amplified by PCR with PpFBXgf7 and PpFBXgr3 primers and digested with *Nsp*I. (b) CAPS analysis of the *S*^{4}-*RNase*. *S-RNase* fragments were amplified by PCR with FTQQYQ and anti-(I/T) IWPNV primers and digested with *Nde*I. *S* haplotype-specific fragments are indicated by arrows and numbers show fragment sizes (bp). (c) CAPS analysis for detection of the S^k haplotype. *PpSFBB*$^{-Y}$ fragments were amplified by PCR with PpFBXgf7 and PpFBXgr3 primers and digested with *Apo*I. 1, Hayatama (S^1S^2); 2, Suisei (S^1S^4); 3, Imamura-aki (S^1S^6); 4, Ichiharawase (S^1S^8); 5, Chojuro (S^2S^3); 6, Nijisseiki (S^2S^4); 7, Hosui (S^3S^5); 8, Niitaka (S^3S^9); 9, Kosui (S^4S^5); 10, Shinkou (S^4S^9); 11, Okusankichi (S^5S^7); 12, Kinchaku (S^4S^k). The S^k haplotype specific fragment is indicated by an *arrow* and the *number* shows the fragment size (bp).

General Discussion and Conclusion

3.1 Analysis of Apple *S* locus

To identify the pollen *S* gene in Maloideae, a subfamily of Rosaceae, 317-kb region of apple S^9 haplotype was completely sequenced. Several findings showed 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. In addition, 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 size of the *S* locus between species have also 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.

3.2 Characterization of pollen *S* candidate genes

As a result of apple *S* locus GENSCAN annotation, 2 ORFs showed homology to *Prunus SLFL1* from S^9 haplotype. These genes contain F-box motif and named *SFBB* (*S* locus F-box brothers). *SFBB* genes were also isolated from Japanese pear (*PpSFBB*). During preparation of first article (Sassa et al. 2007), Cheng et al. (2006) isolated *S* locus-linked and pollen-expressed F-box genes from apple by PCR and named them as *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. 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. Thus, *SFBB* genes meet the expected characteristics of pollen *S*. Furthermore, this study showed the possibility that *SFBB* genes have unique feature, multiplicity. An interesting possibility is that SFBB proteins form a multimer in pollen as suggested by Luu et al. (2001). However, whether all of the multiple *SFBB*s in a haplotype are involved in pollen *S* specificity is not clear, and the possibility that none of them are pollen determinant can not be excluded at present. Recently, many pollen-expressed F-box proteins around the *S* locus were found in *Nicotina alata* (authors designed *DD1-10*; Wheeler and Newbigin 2007). Further analysis of *SFBB* and *DD* genes are required to identify pollen *S* gene.

Sequence polymorphism of *PpSFBB*$^{-Y}$ genes were analyzed in Japanese pear. Among the *PpSFBB* groups, *PpSFBB*$^{-Y}$ genes are most suspicious group for the pollen *S* because of *PpSFBB*$^{-Y}$ genes have high sequence similarity between S^4 and S^5 haplotype (99%, Table 1.5). I isolated new seven *SFBB*$^{-Y}$ genes from different *S* genotypes of Japanese pear. These genes showed *S* haplotype-specific polymorphisms although sequence similarities among them were extremely high. Thus, the possibility that *PpSFBB*$^{-Y}$ genes are involved in pollen *S* specificity cannot be excluded.

3.3 Establishment of new *S* genotyping system

Based on the sequence polymorphisms of the *SFBB*$^{-Y}$ genes, I developed a CAPS/dCAPS system for *S* genotyping of the Japanese pear cultivars. This new *S* genotyping system was found to not only be able to discriminate the S^1-S^9 haplotype, but also be suitable for identification of the mutant S^{4sm} haplotype for the breeding of self-compatible cultivars, and detection of new *S* haplotypes such as S^k.

3.4 Different models and probable mechanistic diversity of the S-RNase based GSI systems

The multiplicity of *SFBB* may suggest that Maloideae has unique self-incompatibility mechanism. Moreover, several reports suggest that SI mechanisms are different among plant species. Competitive interaction of pollen *S* has been documented in Maloideae (Crane and Lewis 1941; Lewis and Modlibowska 1942) and Solanaceae (de Nettancourt 2001). Meanwhile, in *Prunus*, the recent finding that *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 Maloideae. However, recent report suggests heteroallelic pollen grains of *Prunus pseudocerasus* lose pollen *S*-specificity, making it difficult to conclude if competitive interaction confers self-compatibility in *Prunus* (Huang et al. 2008). Moreover, even in Solanaceae, different models have been proposed based on different findings, indicating that it is now still far from full understanding of the GSI mechanism and thus difficult to conclude that some observed 'difference' actually represents mechanistic diversity of GSI.

Recent immunolocalization study revealed that S-RNase is sequestered in pollen tube vacuoles and the membrane surrounding the compartment contains HT-B and 120K, known as non-*S* specific factors in *Nicotiana* (Gordraij et al. 2006). It was also revealed that HT-B protein is degraded after compatible pollinations. Based on these findings, the authors proposed the compartmentalization model (Goldraij et al. 2006; McClure 2006). In this model, S-RNases, 120K and HT-B are taken up from the extracellular matrix by endocytosis. In compatible pollinations, pollen overcomes rejection by degrading HT-B and compartmentalizing S-RNase. In incompatible pollinations, the self S-RNase/SLF interaction leads to stabilization of HT-B, compartment breakdown and release of S-RNase. On the other hand, biochemical analysis revealed that S-RNase physically interacts with SLF, and the

intensity of the interaction between nonself S-RNase/SLF was much stronger than that of the self S-RNase/SLF in *Petunia* (Hua and Kao 2006). Moreover, the authors revealed that S-RNases are degraded via the 26S proteasome pathway. The authors proposed the new protein-degradation model based on these results. This model postulates that, in incompatible pollination, SLF interacts weakly with self S-RNase, and thus most of the S-RNase molecules taken up by the pollen tube and located in the cytoplasm are free to degrade RNA. In compatible pollination, SLF interacts strongly with its nonself S-RNase and the interaction results in the degradation of S-RNase via ubiquitin 26S proteasome pathway.

Compartmentalization model is derived from cell biological study and explains the roles of non-*S* specific factors, however, it is not clear how the S-RNase/SLF interaction controls HT-B degradation and membrane breakdown. Role of the HT-B in membrane destabilization is also hypothetical. On the other hand, the protein-degradation model proposed by Hua et al. (2006) explains biochemical basis of the S-RNase based self-incompatibility. However, no *in vivo* evidence for the proposed model has been presented yet. In addition, the roles of the non-*S*-specific factors are not taken into consideration in this model. The apparent discrepancy on the model of the S-RNase based GSI of Solanaceae suggests that some important pieces are still missing to complete the puzzle. Therefore, at present, it seems difficult to conclude whether S-RNase based self-incompatibility mechanisms are different among species.

Rosaceae belongs to subclass Rosidae and is distantly related to Solanaceae and Plantaginaceae that belong to subclass Asteridae. Elucidation of the self-incompatibility mechanism in Maloideae, especially for the identification of the pollen *S* gene, will provide valuable insights into the origin and diversity of S-RNase based self-incompatibility. Wang et al. (2008) recently reported that pollen tube after incompatible pollination showed the features of programmed cell death (PCD) in Japanese pear. It has not been reported that GSI

of Solanaceae or Plantaginaceae plants are related to PCD. It may be important to examine whether PCD is occur in other families. However, it is not clear whether PCD plays a pivotal role in GSI or just a result of the incompatible reaction in pear. It is expected that functional analysis of *SFBB*s, and cell biological and biochemical studies will shed light on the self-incompatibility mechanism in Maloideae. Full understanding of the GSI of Maloideae will not only be valuable for breeding of the self-compatible cultivar but also important for elucidation of the origin and diversity of the S-RNase based GSI mechanism.

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List of achievements

Publications

1) Sassa H, Kakui H＊, Miyamoto M, Suzuki Y, Hanada T, Ushijima K, Kusaba M, HiranoH, Koba T (2007) *S*-locus F-box brothers: Multiple and pollen-specific F-box genes with*S*-haplotype-specific polymorphisms in apple and Japanese pear. Genetics 175:1855-1867 ＊equally contributed as the first author

2) Kakui H, Tsuzuki T, Koba T, Sassa H (2007) The polymorphism of *SFBB*$^{\gamma}$ and its use for *S* genotyping in Japanese pear (*Pyrus pyrifolia*). Plant Cell Rep 26:1619-1625

3) Kim HT, Kakui H, Koba T, Hirata Y, Sassa H (2007) Cloning of a new *S-RNase* and development of a PCR-RFLP system for determination of the *S*-genotypes of Japanese pear. Breeding Sci 57: 159-164

4) Kim HT, Kakui H, Kotoda N, Hirata Y, Koba T, Sassa H (2009) Determination of partial genomic sequences and development of a CAPS system of the *S-RNase* gene for identification of 22 *S* haplotypes of apple (*Malus* × *domestica* Borkh.) Mol Breeding ; Accepted

Research fellowship

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Oral presentations （○：発表者）

1) ○角井宏行・宮本摩由・佐々英徳・平野久、「ニホンナシの花粉特異的 F-box 遺伝子」、『日本育種学会』、417、筑波大学、2005 年 8 月

2) 角井宏行・○佐々英徳・平野久、「ニホンナシの花粉で発現する *Skp1* 様遺伝子の単離」、『日本育種学会』、418、筑波大学、2005 年 8 月

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