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Apple *S* locus region represents a large cluster of related, polymorphic and pollen-specific F-box genes

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Abstract Gametophytic self-incompatibility (GSI) of Rosaceae, Solanaceae and Plantaginaceae is controlled by a complex *S* locus that encodes separate proteins for pistil and pollen specificities, extracellular ribonucleases (S-RNases) and F-box proteins SFB/SLF, respectively. *SFB/SLF*s of *Prunus* (subfamily Prunoideae of Rosaceae), Solanaceae and Plantaginaceae are single copy in each *S* haplotype, while recently identified pollen *S* candidates *SFBB*s of subfamily Maloideae of Rosaceae, apple and Japanese pear, are multiple; two and three related *SFBB*s were isolated from each *S* haplotype of apple and Japanese pear, respectively. Here, we show that apple (*Malus* × *domestica*) *SFBB*s constitute a gene family that is much larger than initially thought. Twenty additional *SFBB*-like genes/alleles were isolated by screening of a BAC library derived from S^3S^9 genotype, and tentatively named *MdFBX1-20*. All but one *MdFBX* showed *S* haplotype-specific polymorphisms. All the polymorphic *MdFBX*s were completely linked to *S-RNase* in 239 segregants. In addition, FISH revealed that the monomorphic gene *MdFBX11* is also located near *S-RNase*, and the *S* locus is located in a subtelomeric region of a chromosome and is not close to the centromere. All *MdFBX*s were specifically expressed in pollen, except for a pseudogene *MdFBX4* that showed no expression in any organs analyzed. Phylogenetic analysis revealed that the closest relatives of most *MdFBX*s were from a different *S* haplotype, suggesting that proliferation of *MdSFBB/FBX*s predates diversification of the *S* haplotypes.

Keywords Self-incompatibility · *S* locus · Rosaceae · F-box · Pollen

Abbreviations

GSI	Gametophytic self-incompatibility
SFBB	*S* locus F-box brothers
S-RNase	S-ribonuclease
BAC	Bacterial artificial chromosome

Sequence data from this article have been deposited with DDBJ/EMBL/GenBank Data Libraries under accession nos. AB539844 (*MdFBX1*), AB539845 (*MdFBX2*), AB539846 (*MdFBX3*), AB539847 (*MdFBX4*), AB539848 (*MdFBX5*), AB539849 (*MdFBX6*), AB539850 (*MdFBX7*), AB539851 (*MdFBX8*), AB539852 (*MdFBX9*), AB539853 (*MdFBX10*), AB539854 (*MdFBX11*), AB539855 (*MdFBX12*), AB539856 (*MdFBX13*), AB539857 (*MdFBX14*), AB539858 (*MdFBX15*), AB539859 (*MdFBX16*), AB539860 (*MdFBX17*), AB539861 (*MdFBX18*), AB539862 (*MdFBX19*), AB539863 (*MdFBX20*).

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Introduction

Self-incompatibility (SI) is a genetic mechanism to prevent inbreeding and to promote outcrossing found in many

flowering plants. 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 control the specificity of self/non-self 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 tightly linked *S*-determinants, pistil *S* and pollen *S* (Kao and Tsukamoto 2004; Franklin-Tong 2008).

Pistil *S* is known as the *S-RNase* gene which encodes an extracellular ribonuclease (Anderson et al. 1986; McClure et al. 1989; Sassa et al. 1996, 1997; Tao et al. 1997; Xue et al. 1996). S-RNases are taken up by both compatible and incompatible pollen tubes, but thought to act as cytotoxins only in incompatible pollen tubes to arrest growth (Goldraij et al. 2006; Luu et al. 2000). The identification of F-box genes as pollen *S* (candidates), *SLF* (*S* locus F-box)/*SFB* (*S* haplotype-specific F-box), raised the possibility that ubiquitin/proteasome pathway plays a pivotal role in GSI because the predominant function of F-box protein is substrate recognition as a component of SCF complex, a kind of E3 ubiquitin ligase (Entani et al. 2003; Lai et al. 2002; Qiao et al. 2004a; Sijacic et al. 2004; Ushijima et al. 2003, 2004; Wang et al. 2004). It has been hypothesized that SLF/SFB recognizes non-self S-RNase in compatible pollen tubes and ubiquitinates it for degradation by the 26S proteasome (Hua and Kao 2006; Hua et al. 2008; Qiao et al. 2004b; Ushijima et al. 2003, 2004). However, Goldraij et al. (2006) showed that S-RNase is sequestered into vacuoles in pollen tubes and that the amount of S-RNase is not significantly different between compatible and incompatible pollination. Consequently, how SLF/SFB and S-RNase interact with each other to trigger the self-incompatibility reaction in vivo remains largely unclear. Furthermore, genetic studies suggested divergence of pollen *S* function among taxa.

In Solanaceae and subfamily Maloideae of Rosaceae (e.g., apple and pear), it has been well-documented that coexistence of different pollen *S* in a pollen grain as a result of polyploidization or translocation of the *S* locus causes breakdown of SI in pollen leaving the pistil SI function unaffected (Adachi et al. 2009; Crane and Lewis 1941; de Nettancourt 2001; Golz et al. 2001; Lewis and Modlibowska 1942). This phenomenon, known as 'competitive interaction', was recently reported also in Plantaginaceae, *Antirrhinum* (Xue et al. 2009). In contrast, in species of the subfamily Prunoideae of Rosaceae (e.g., almond and sweet cherry), it is suggested that the pollen *S* gene does not cause competitive interaction. Sour cherry (*Prunus cerasus*) is a naturally occurring tetraploid species and includes both SI and self-compatible (SC) lines (Lansari and Iezzoni 1990). Hauck et al. (2006b) conducted genetic analyses of sour cherry and proposed that the breakdown of SI is caused by the accumulation of non-functional *S* haplotypes and not by competitive interaction in heteroallelic pollen. Consistent with this, different pollen-part SC mutants of *Prunus* contained *SFB* genes encoding truncated proteins (Hauck et al. 2006a; Tsukamoto et al. 2005; Ushijima et al. 2004). Furthermore, Sonneveld et al. (2005) showed complete deletion of *SFB* in a SC haplotype of sweet cherry. This is in marked contrast to the case of Solanaceae, in which pollen *S* is considered to be required for pollen tube growth. In *Nicotiana* (Solanaceae), no pollen *S* deletion type mutant but pollen *S* duplication types were recovered after large scale screening of mutagenized pollen by incompatible pollination, suggesting that pollen *S* is essential for pollen tube growth (Golz et al. 2001). Pollen *S* deletion type mutant is not known in Maloideae either.

Consistent with the probable difference in pollen *S* function between Maloideae and Prunoideae, recently identified pollen *S* candidates of Maloideae show a unique feature, multiplicity (Sassa et al. 2010). Two related F-box genes were identified as pollen *S* candidates by sequencing of the *S* locus region of apple (*Malus* × *domestica*) and named *SFBB*s (*S* locus F-box brothers; Sassa et al. 2007). Three *SFBB* genes were isolated by PCR from each *S* haplotype of Japanese pear (*Pyrus pyrifolia*). All the *SFBB*s showed *S* haplotype-specific polymorphisms, linkage to the *S* locus and pollen-specific expression. Here, we show that many more *SFBB*-like genes are clustered in the *S* locus region of apple. We isolated twenty *SFBB*-like genes from apple with S^3S^9 genotype, and tentatively named them *MdFBX1-20*. Linkage to the *S-RNase* gene, *S* haplotype-specific polymorphisms and pollen-specific expression were found in all the *MdFBX* genes, except for a pseudogene and a monomorphic gene. We discuss the implications of the *MdSFBB/FBX* cluster at the apple *S* locus for the probable divergence of the GSI mechanism among taxa.

Materials and methods

Plant material

Eight apple cultivars, Sekai-ichi (S^3S^9), Natsumidori (S^3S^9), Kitaro (S^3S^9), Hatsuaki (S^3S^9), Golden Delicious (S^2S^3), Megumi (S^2S^9), Gala (S^1S^6) and Morioka64 (S^3S^5), were used. 175 progenies obtained by crossing Kitaro (S^3S^9) and Morioka64 (S^3S^5) and 64 progenies obtained by crossing Hatsuaki (S^3S^9) and Megumi (S^2S^9) were used to analyze the linkage of F-box genes with the *S-RNase* gene. Seedlings with S^9 haplotype that were derived from open pollinated Fuji (S^1S^9) fruits were selected by PCR (Kim

et al. 2009) and used for fluorescence in situ hybridization (FISH) analysis.

Isolation of nucleic acids

Genomic DNA was isolated from leaves as described by Sassa (2007). RNA was isolated from leaves and floral organs as described by McClure et al. (1990).

Screening of a BAC library and cloning of *SFBB*-like genes

A BAC library of an apple cultivar, Florina, covering five genome equivalents (Vinatzer et al. 1998) was obtained from Texas A&M University and screened using $MdSFBB^{9-\beta}$ (Sassa et al. 2007) as a probe. *SFBB*-like genes were amplified from the selected BAC clones using the primers FSMF1 and RSMF1(Sassa et al. 2007). A standard PCR mixture (in 20 µl total volume) included BAC plasmid, 0.4 µM of each primer, 200 µM dNTP, 1× PCR Buffer, and 0.5 U of ExTaq (TaKaRa). Amplification was carried out for 35 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, and extension at 72°C for 1.25 min, with a final extension at 72°C for 7 min. The PCR products were cloned into a plasmid vector and sequenced. For three genes that were not amplified from BAC clones with FSMF1 and RSMF1, partial sequences were amplified by different primer pairs: FjpFB1 (Sassa et al. 2007) and RSMF1 for *MdFBX17* and *19*, and aBACf6 and aBACr8 for *MdFBX20*. Flanking sequences were determined by using DNA Walking SpeedUp Premix Kit (SeeGene) or by 5′RACE. The primer sequences used for amplification of those three genes are listed in Supplemental Tables 1 and 2. Sequence data was analyzed with BioEdit ver.7.0 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and ClustalW (Thompson et al. 1994). The identity of amino acid sequences of 20 *MdFBX*s was examined using GENETYX-MAC program (GENETYX Co. Tokyo, Japan).

CAPS and dCAPS analyses

Genomic DNA of apple cultivars was used as the template for amplification of *MdFBX* genes. The PCR products were digested with restriction enzymes to detect specific cleaved amplified polymorphic sequence (CAPS) bands. Derived CAPS (dCAPS; Neff et al. 1998) analysis was conducted to detect *MdFBX10, 15* and *20*-specific fragments. The primers and enzymes are listed in Supplemental Table 1. The products were separated on 1 or 2% agarose gels and visualized by staining with ethidium bromide. CAPS analysis of the *S-RNase* genes was conducted according to the procedure of Kim et al. (2009). PCR products were treated with *Sph*I to differently digest S^3-, S^5- and S^9-*RNase* fragments (Kim et al. 2009).

FISH

Root tips of seedlings with S^9 haplotype derived from Fuji (S^1S^9) were pretreated with 2 mM 8-hydroxyquinoline for 2 h at room temperature and 4 h at 4°C, and fixed in 3:1 (v/v) ethanol: acetic acid for >24 h. After washing in distilled water, chromosome slides were prepared by enzymatic maceration (2% cellulase and 2% pectolyase Y-23) at 37°C for 20 min and squash method. The slides were incubated in 100 ng/µl RNase for 1 h at 37°C in a wet chamber, and then incubated in 5 ng/µl pepsin for 20 min at 37°C, followed by post-fixation in 3:1 (v/v) ethanol: acetic acid for 5 min. BAC clones were labeled with either biotin-16-dUTP or digoxigenin-11-dUTP by nick translation and used as FISH probes. A 10 µl hybridization solution (50% formamide, 10% dextran sulfate, 2× SSC, 20 ng of each probe, 3.0 µg of apple genomic blocking DNA) was applied to the slides. Chromosomal DNA was denatured in the hybridization solution for 5 min at 80°C and then kept in the chamber at 37°C for 2 days. After washing in distilled water, FITC-conjugated streptavidin and mouse anti-digoxigenin were applied to slide and incubated for 30 min at 37°C. After washing, Alexa fluor 568-conjugated rabbit anti-mouse IgG (H + L) was applied as secondary antibody and incubated for 30 min at 37°C. Chromosomes were counterstained with 4, 6 diamidino-2- phenylindole (DAPI).

RT-PCR/CAPS

RNA samples from the leaves and the floral organs of Sekai-ichi (S^3S^9) were treated with DNaseI (Nippongene). cDNA was synthesized by ReverTra Ace (TOYOBO) and used for RT-PCR/CAPS. As the control in the RT-PCR, *ef1-α* (translation elongation factor 1α, AY338249) was amplified by the primers ef1-αf1 (5′-ATTGTGGTCATT GYCAYGT-3′) and ef1-αr1 (5′-CCTATCTTGTAVACA TCCTG-3′).

Construction of phylogenetic trees of *S* locus-linked F-box proteins

The amino acid sequences of the *S* locus-linked F-box proteins of Solanaceae, Rosaceae and Plantaginaceae were aligned using ClustalW (Thompson et al. 1994). A neighbor-joining tree was constructed using the alignment (Saitou and Nei 1987). Maximum parsimony phylogenetic tree was generated by MEGA4 (Kumar et al. 2008).

Results

Twenty new *SFBB*-like genes isolated from a BAC library of apple Florina (S^3S^9)

Screening of a Florina (S^3S^9) BAC library covering five × haploid-genome equivalents (Vinatzer et al. 1998) with the $MdSFBB^{9-\beta}$ probe was conducted under low stringent hybridization condition (50°C), and identified 39 positive clones. *SFBB*-like genes were amplified from the positive BAC clones by PCR. Identical sequences were obtained from several clones, probably because the BACs are overlapping. The overlapping of BAC clones was confirmed by fingerprinting the clones with restriction digestion (data not shown). Finally, twenty new *SFBB*-like genes were isolated and tentatively named *MdFBX1* ~*20*. All 20 *MdFBX* genes encoded SFBB-like proteins except for *MdFBX4*, which encoded a truncated protein because of in-frame stop codons, and was considered to be a pseudogene. MdFBXs and previously identified MdSFBBs were highly homologous with each other showing 59.6% (MdFBX14 and MdFBX10) to 98.0% (MdFBX5 and

Fig. 1 Amino acid sequence alignment of the *MdSFBB/MdFBX* genes. Amino acid sequences of MdSFBB/MdFBX proteins were aligned using ClustalW. Conserved sites and relatively conservative sites are marked with asterisks and dots, respectively. The 28th and 70th asterisks of MdFBX4 are premature stop codons. A *double-headed arrow* denotes the F-box region. FBA_1 (F-box associated) motif detected by Pfam (http://pfam.sanger.ac.uk) was denoted by a *line*

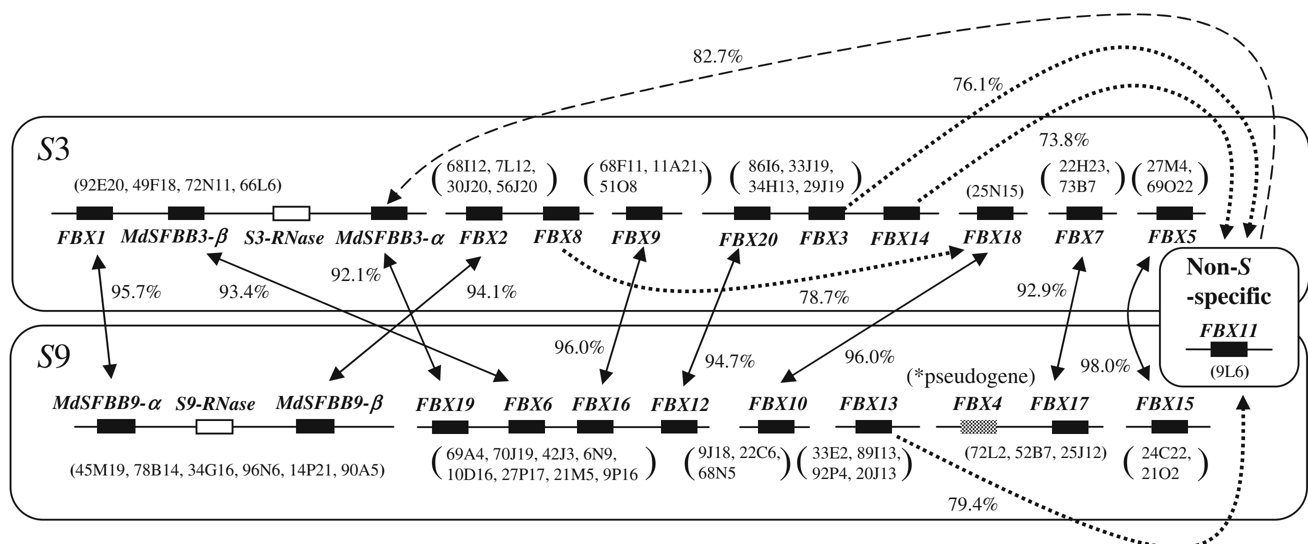


Fig. 2 The BAC contigs for the *S* locus region of apple. *Black and white boxes* denote *MdSFBB/MdFBX* genes and *S-RNase* genes, respectively. *A gray box* represents a pseudogene *MdFBX4*. *Arrows* indicate closest relatives of the *MdSFBB/MdFBX* genes. *Solid*, *dashed* and *dotted lines* denote the amino acid identities ≥90%, 80.0–89.9% and 70.0–79.9%, respectively. BAC clones that constitute the contigs were in the *parentheses*. Relative order and orientation of BAC contigs are not determined. *Bars* are not in scale

MdFBX15) amino acid sequence identity (Figs. 1, 2; Supplemental Table 3). All the MdSFBB/FBXs contained FBA_1 motif (F-box associated) in addition to the N-terminal F-box region. No intron was found in all the *MdSFBB/FBX* genes.

S haplotype-specific sequence polymorphisms of *MdFBXs*

Since the *SFBB/FBX* genes are similar with each other (Sassa et al. 2007; Supplemental Table 3), cleaved amplified polymorphic sequence (CAPS) and derived cleaved amplified polymorphic sequence (dCAPS) procedures were used to determine whether the newly identified *MdFBX*s show *S* haplotype-specific polymorphisms. CAPS and dCAPS analyses were conducted using different *S* genotypes and showed that all twenty genes were correlated with *S* haplotypes except for *MdFBX11*. Ten genes (*MdFBX1,2,3,5,7, 8,9,14,18* and *20*) were associated with the S^3 haplotype, while nine genes (*MdFBX4,6,10,12,13,15,16,17* and *19*) were specific to the S^9 haplotype (Supplemental Fig. 1; Fig. 2). *S* haplotype-specific sequence motif was not detected in those F-box genes. The sequences of *MdFBX11* derived from two genotypes (S^2S^5 and S^3S^9) were completely identical (data not shown), showing that *MdFBX11* is a monomorphic gene.

MdFBX genes are linked to *S-RNases*

Two segregating populations derived from a cross between Kitaro (S^3S^9) × Morioka64 (S^3S^5) and Hatsuaki (S^3S^9) × Megumi (S^2S^9) were analyzed by the CAPS/dCAPS system to examine linkage between *MdFBX*s and *S-RNase* genes. Figure 3 shows representative results of the linkage analysis for the Kitaro (S^3S^9) × Morioka64 (S^3S^5) population. Analysis of the *S-RNase* gene of the two populations showed that genotypes of Kitaro (S^3S^9) × Morioka64 (S^3S^5) were segregated as S^3S^5:S^5S^9 = 92:83, while Hatsuaki (S^3S^9) × Megumi (S^2S^9) resulted in S^2S^3:S^2S^9 = 30:34. CAPS/dCAPS analysis of *MdFBX*s showed that all S^3-associated genes (*MdFBX1,2,3,5,7,8,9,14,18* and *20*) were completely linked to S^3-*RNase* in a total of 239 plants., i.e., the S^3-associated *MdFBX* genes were detected in 92 S^3S^5 plants and 30 S^2S^3 plants but not in other plants with the S^5S^9 or S^2S^9 genotype. Similarly, complete linkage between S^9-associated genes (*MdFBX4,6,10,12,13,15,16,17* and *19*) and S^9-*RNase* was observed in the 239 plants.

Because *MdFBX11* was monomorphic and cannot be subjected to genetic analysis, linkage of *MdFBX11* to the *S* locus was examined by FISH analysis. First, two BAC clones derived from the *S* locus, BAC 45M19 containing $SFBB^{9\alpha}$ and BAC 34G16 containing S^9-*RNase* (Sassa et al. 2007), were used as FISH probes to examine the chromosomal location of the *S* locus. 45M19 was clearly detected in the subtelomeric region of a chromosome and overlapped with 34G16 (Fig. 4a). In addition, the signals of 34G16 were observed at centromeric regions of each chromosome, probably because 34G16 contains repetitive sequence(s) that is/are abundant in the pericentromeric region. This finding suggests that the *S* locus is not localized at the pericentromeric region. Next, the chromosomal position of monomorphic *MdFBX11* was analyzed. BAC 45M19 as the

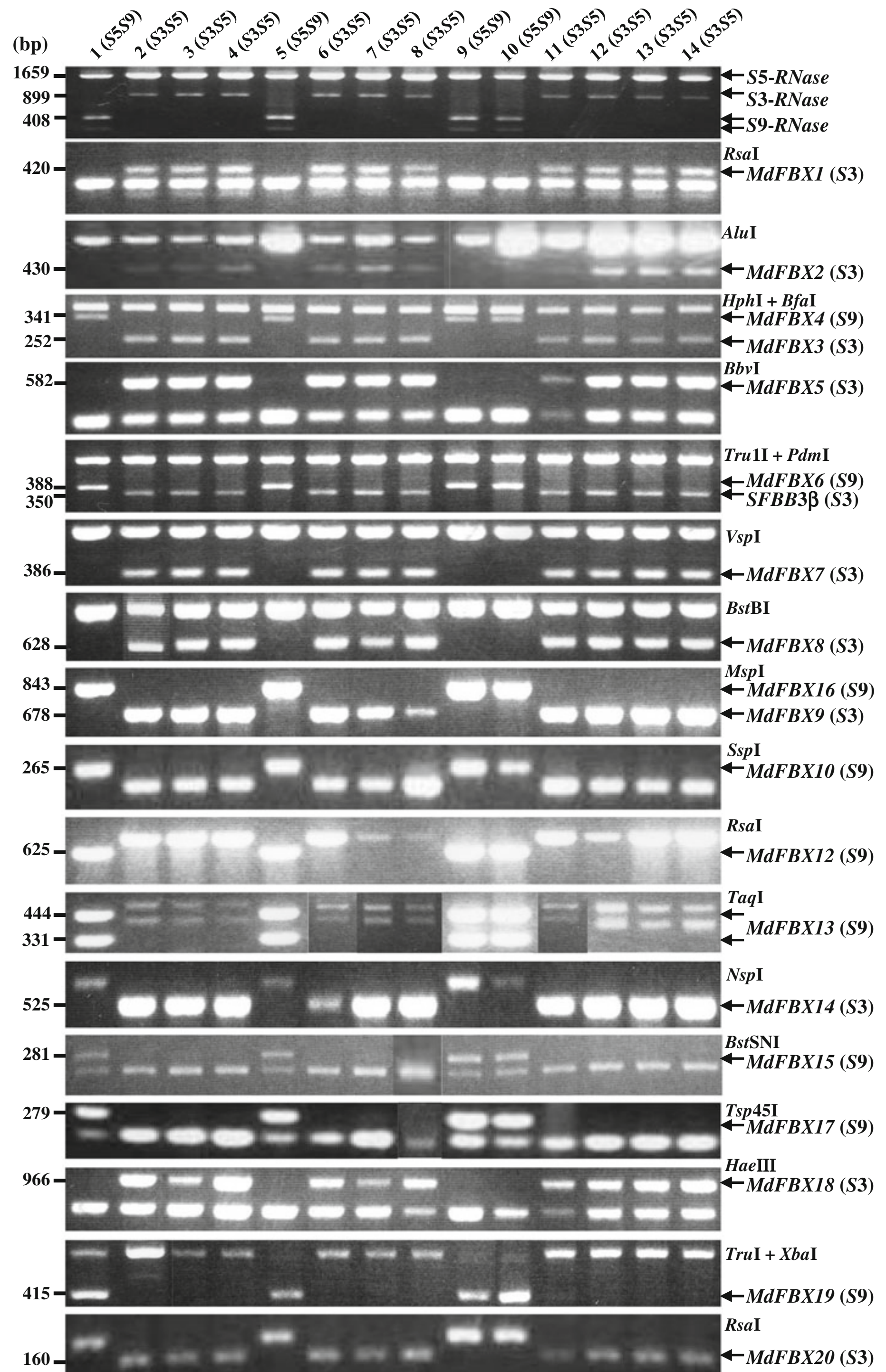


Fig. 3 Linkage analysis of the *MdFBX*s and the *S-RNase*s of apple. 239 progenies from Kitaro (S^3S^9) × Morioka64 (S^3S^5) and Hatsuaki (S^3S^9) × Megumi (S^2S^9) were analyzed using CAPS. Representative results of Kitaro (S^3S^9) × Morioka64 (S^3S^5) population were shown

S locus probe and BAC 9L6 as the *MdFBX11* probe were hybridized to chromosomal DNA. FISH showed that *MdFBX11* was located near the *S* locus (Fig. 4b). The FISH result was confirmed to be reproducible in repeated experiments ($n = >50$).

MdFBX genes are specifically expressed in pollen

To examine the organ-specific expression of *MdFBX* genes, RT-PCR followed by CAPS analysis (RT-PCR/CAPS) was conducted. For *MdFBX17*, restriction digestion

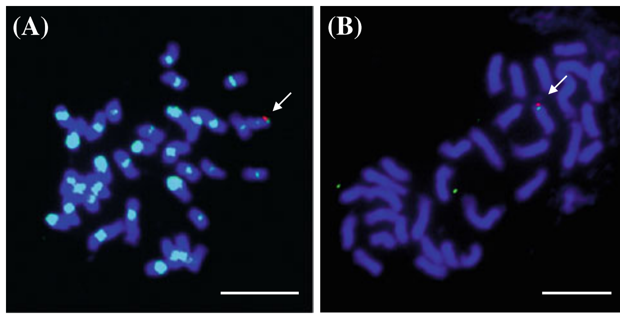


Fig. 4 FISH mapping of BAC clones including *S^9-RNase*, *$MdSFBB^{9-\alpha}$* and *MdFBX11* on chromosomal DNA. *Bars* = 10 µm. *Arrows* indicate the *S* locus region. **a** BAC 45M19 containing *$SFBB^{9-\alpha}$* (*red*; Alexa fluor 568) was located in a subtelomeric region of a chromosome and overlapped with 34G16 containing *S^9-RNase* (*green*; FITC). **b** The green signal (FITC) of BAC 9L6 containing *MdFBX11* was located near the *red signal* (Alexa fluor 568) of 45M19

of the RT-PCR product was omitted because it was found to be specifically amplified by the RT-PCR condition. The monomorphic gene *MdFBX11* was also analyzed by RT-PCR. Results showed that all the *MdFBX* genes, except for the pseudogene *MdFBX4*, were specifically expressed in pollen (Fig. 5). Expression of the pseudogene *MdFBX4* was not observed in any organs analyzed.

Phylogenetic analysis of MdFBXs

The full length amino acid sequences of the *S* locus linked F-box proteins of Rosaceae, Solanaceae and Plantaginaceae were aligned and used to construct a phylogenetic tree using the neighbor-joining method with *Arabidopsis thaliana S* locus SLF-related F-box (NP_180975) as an outgroup (Fig. 6). SFBBs/FBXs of Maloideae formed a well-supported (bootstrap value = 100%) compact clade. In *Prunus*, F-box proteins formed three distinct clades; SFBs, SLFL2, and SLFL1 and 3. SFBBs/FBXs were more similar to probable non-*S* F-box proteins SLFL1 and 3 of *Prunus* than to *Prunus* pollen *S* proteins SFBs as previously suggested (Matsumoto et al. 2008; Sassa et al. 2007). Topology of maximum parsimony tree was similar to that of neighbor-joining tree (Supplemental Fig. 2).

For most, if not all, SFBBs/FBXs, closest relatives were of different haplotypes, e.g., MdFBX9 (S^3) was closest to MdFBX16 (S^9) (Fig. 6; Supplemental Table 3). Relative position was, however, not same in some closely related gene pairs (Fig. 2). For example, two F-box genes *$SFBB^{3-\alpha}$* and *$SFBB^{3-\beta}$* are proximately localized to the *S^3-RNase*, while their closest relatives, *FBX19* and *FBX6*, respectively, are not located beside the *S^9-RNase*. Similarly, *$SFBB^{9-\beta}$* is proximately localized to the *S^9-RNase*, while its closest relative, *FBX2*, is not located in the *S^3-RNase*-containing BAC contig.

Discussion

Many *SFBB*-class F-box genes are clustered in the *S* locus region in apple

In different species of Rosaceae, Solanaceae and Plantaginaceae, the *S* locus region contains several F-box genes, the pollen *S* genes *SLF/SFB*s, and other (probable) non-*S* F-box genes *SLFL*s (Lai et al. 2002; Entani et al. 2003; Ushijima et al. 2003; Yamane et al. 2003; Zhou et al. 2003; Wang et al. 2004; Tsukamoto et al. 2005). Pollen *S* F-box genes are less homologous to non-*S* F-box genes, and are single copy in *S* haplotypes. In contrast, a unique feature of the pollen *S* candidate *SFBB* of Maloideae, apple and pear, is that related and multiple genes are located at the *S* locus. Two and three *SFBB*s were identified in each *S* haplotype of apple and Japanese pear, respectively (Sassa et al. 2007).

In this study, we conducted a comprehensive analysis of the *SFBB* genes of apple and showed that the *SFBB* family is larger than initially thought. We identified twenty additional *SFBB*-like genes/alleles that were provisionally named *MdFBX*s, and showed that all of them are linked to the *S* locus. Ten and nine *MdFBX*s were genetically linked to the S^3- and *S^9-RNase*, respectively. FISH analysis showed physical linkage of monomorphic *MdFBX11* to the *S* locus. Although the possibility that several *S* locus-linked *SFBB/FBX* genes remain to be identified can not be excluded, because we didn't determine complete sequences of the isolated BAC clones, this study revealed that the *S* locus region of apple represents the largest F-box gene cluster known, composed of more than ten highly homologous members.

CAPS/dCAPS and sequence comparison analyses revealed that all *SFBB/FBX* genes showed *S* haplotype-specific polymorphisms, except for *MdFBX 11*. A monomorphic F-box gene that is highly homologous to pollen *S* was also found in *Petunia* and named *PaF1*, although linkage of *PaF1* to the *S* locus is unclear (Tsukamoto et al. 2005). Phylogenetic analysis revealed that SFBB/FBX proteins didn't form *S* haplotype-specific groups, and, instead, closest relatives were from different *S* haplotypes for most, if not all, proteins. This finding suggests that each closely related gene pair shares a common ancestor, and proliferation of the *SFBB/FBX* genes at the *S* locus predates diversification of the *S* haplotypes. Relative order of the genes was not conserved in some closely related gene pairs, e.g., for two F-box genes that are closely localized to the *S^3-RNase*, *$SFBB^{3-\alpha}$* and *$SFBB^{3-\beta}$*, their closest relatives, *FBX19* and *FBX6*, respectively, are not located corresponding position of the S^9 haplotype, but are located outside the BAC contig containing the *S^9-RNase*. In addition, highly similar partner (≥90% amino acid identity) was not detected in some *FBX* genes, suggesting that order

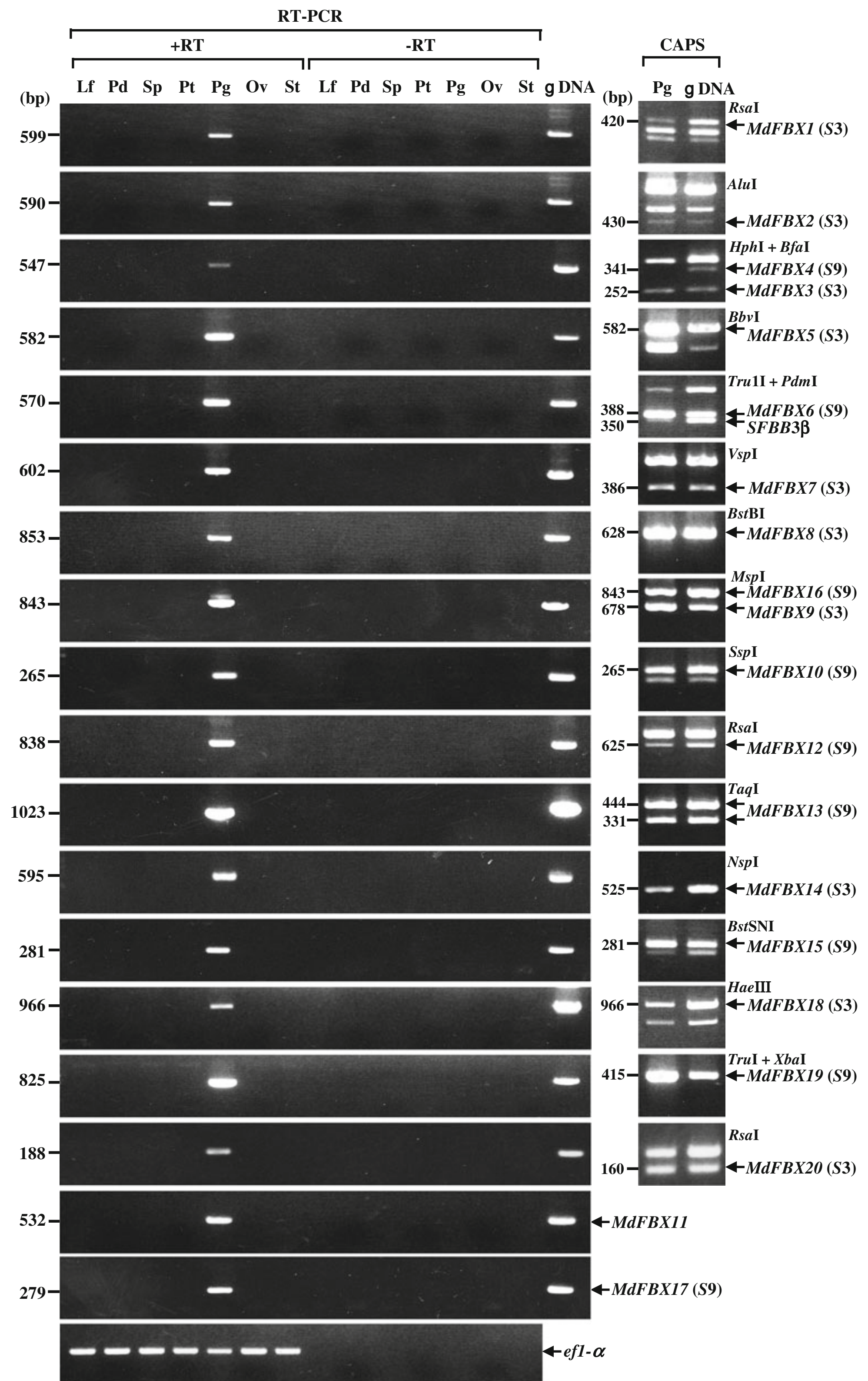
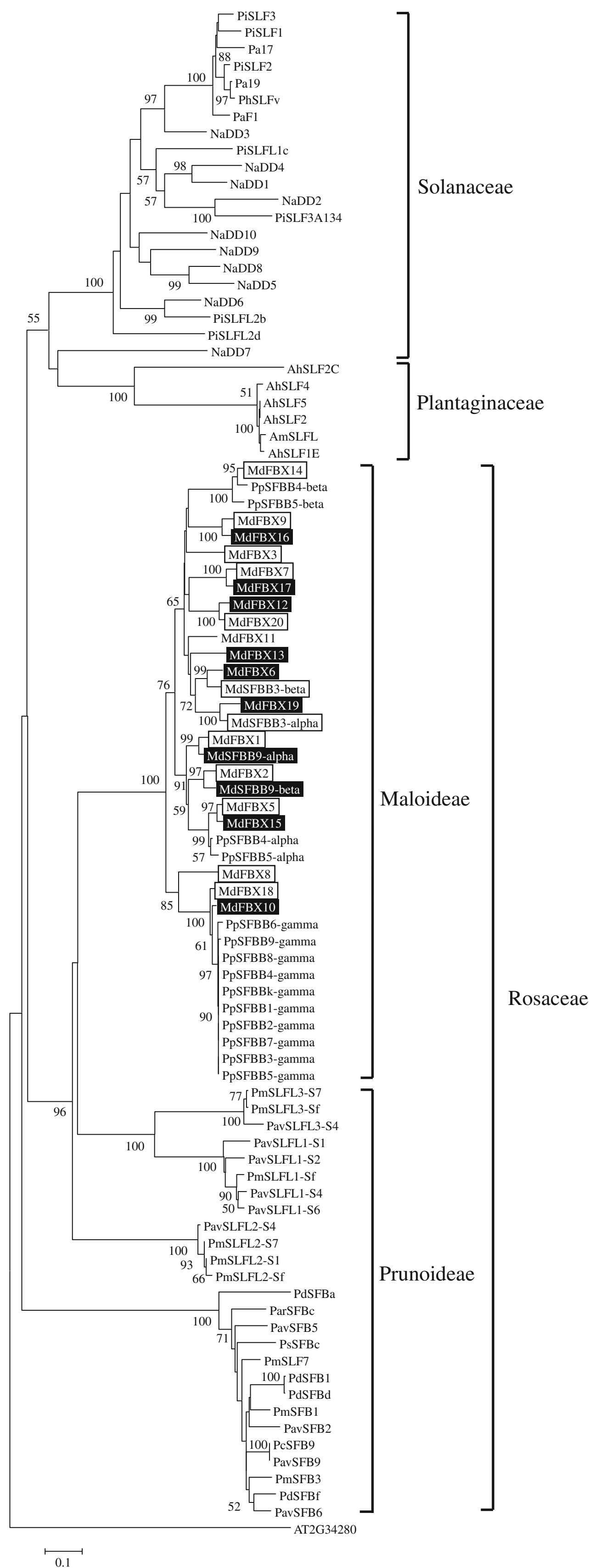


Fig. 5 RT-PCR/CAPS analysis of the expression of *MdFBX*s in different organs. *MdFBX17* was specifically amplified by the RT-PCR condition and thus the restriction digestion step was omitted. Monomorphic *MdFBX11* was also analyzed by RT-PCR. *Lf* leaf, *Pd* pedicel, *Sp* sepal, *Pt* petal, *Pg* pollen grain, *Ov* ovary, *St* style, *gDNA* genomic DNA(S^3S^9)

and repertoire of *SFBB/FBX* genes were rearranged during the course of evolution. The relationship between the molecular mechanism of the GSI of apple and selective pressure that favored proliferation of the *SFBB/FBX* genes at the *S* locus region would be an intriguing issue to be addressed.



◀ **Fig. 6** Neighbor-joining tree of the *S* locus-linked F-box proteins. Abbreviations for the F-box proteins are Md, apple (*Malus* × *domestica*); Pp, Japanese pear (*Pyrus pyrifolia*); Pd, almond (*Prunus dulcis*); Pm, Japanese apricot (*Prunus mume*); Pav, sweet cherry (*Prunus avium*); Pc, sour cherry (*Prunus cerasus*); Ps, Japanese plum (*Prunus salicina*); Par, apricot (*Prunus armeniaca*); Pi, *Petunia inflata*; Ph, *Petunia hybrida*; Pa, *Petunia axillaris*; Na, *Nicotiana alata*; Ah, *Antirrhinum hispanicum*; Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*. Numbers in the phylogenetic tree indicate the percentage of 100 bootstrap replicates in which a group was found (values <50% not shown). MdSFBBs/FBXs from the S^3 haplotype are boxed while MdSFBBs/FBXs from the S^9 haplotype are shaded in *black*. The alignment used to generate the phylogenetic tree is shown in Supplemental Fig. 3. Accession numbers: MdSFBB3-alpha(AB270795), MdSFBB3-beta(AB270796), MdSFBB9-alpha(AB270793), MdSFBB9-beta(AB270794), PpSFBB1-gamma(AB297933), PpSFBB2-gamma(AB297934), PpSFBB3-gamma(AB297935), PpSFBB4-alpha(AB270797), PpSFBB4-beta(AB270798), PpSFBB4-gamma (AB270799), PpSFBB5-alpha(AB270800), PpSFBB5-beta(AB270801), PpSFBB5-gamma(AB270802), PpSFBB6-gamma(AB297936), PpSFBB7-gamma(AB297937), PpSFBB8-gamma(AB297938), PpSFBB9-gamma(AB297939), PpSFBBk-gamma(AB297940), PdSFB1 (FJ514938), PdSFBa(AB092966), PdSFBd(AB081648), PdSFBf (AB361036), PcSFB9(DQ827717), PsSFBc(DQ849084), ParSFBc (DQ422946), PavSFB2(AB111519), PavSFB5(AB111520), PavSFB6(AB096858), PavSFB9(DQ422809), PavSLFL1-S1(AB360339), PavSLFL1-S2(AB360340), PavSLFL1-S4(AB280953), PavSLFL1-S6(AB360342), PavSLFL2-S4(AB280954), PavSLFL3-S4(AB280955), PmSFB1(AB101440), PmSFB3(AB376968), PmSLF7(AB092622), PmSLFL1-Sf(AB280956), PmSLFL2-S1(AB092625), PmSLFL2-S7(AB092626), PmSLFL2-Sf(AB280957), PmSLFL3-S7(AB092627), PmSLFL3-Sf(AB280958), PhSLFv(FN429077), PiSLF1(AY500390), PiSLF2(AY500391), PiSLF3(AY500392), PiSLF3A134(AY363975), PiSLFL1c(EF614191), PiSLFL2b(EF614188), PiSLFL2d(EF614187), Pa17(AY766153), Pa19(AY766154), PaF1(AY766155), NaDD1(EF420251), NaDD2(EF420252), NaDD3(EF420253), NaDD4(EF420254), NaDD5(EF420255), NaDD6(EF420256), NaDD7(EF420257), NaDD8(EF420258), NaDD9 (EF420259), NaDD10(EF420260), AhSLF1E(AJ515535), AhSLF2(CAC33022), AhSLF2C(DQ462204), AhSLF4(AJ515534), AhSLF5(AJ515536), AmSLFL(DQ862124)

Multiple, related, polymorphic and pollen-specific *SFBB/FBX* genes and the GSI mechanism

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; Yamane et al. 2003; Zhou et al. 2003; Wang et al. 2004; Tsukamoto et al. 2005). However, those non-*S* F-box genes, *SLFL*s, are less homologous to the pollen *S* genes *SLF/SFB*s and some of them are also expressed in other organs. Hua et al. (2007) showed that *SLFL*s can not substitute the pollen *S* function of *SLF* in transgenic *Petunia*. Although a recent study on *Nicotiana* identified many *S* locus-linked *SLF*-like genes named *DD*s, the *DD* group is composed of highly divergent genes and some of them are located outside the functional *S* locus, expressed in non-*S* specific manner,

and/or expressed not only in pollen but also in other organs (Wheeler and Newbigin 2007), suggesting that many of the *DD*s correspond to *SLFL* genes of *Nicotiana*. In contrast, a unique feature of the *S* locus of Maloideae is that it contains related and multiple pollen *S* candidates, *SFBB/FBX* genes. The feature of *SFBB/FBX*s might be related to the probable difference in pollen *S* function between two subfamilies of Rosaceae, Maloideae (*Malus* and *Pyrus*) and Prunoideae (*Prunus*).

Loss of SI in heteroallelic pollen has been known as the result of competitive interaction of pollen *S* alleles in Maloideae (Adachi et al. 2009; Crane and Lewis 1941; Lewis and Modlibowska 1942), and also in Solanaceae and Plantaginaceae (de Nettancourt 2001; Xue et al. 2009), while genetic studies suggested that it doesn't occur in *Prunus* (Hauck et al. 2006b; Yamane and Tao 2009). Furthermore, pollen *S* is considered to be essential for pollen tube growth in *Nicotiana* (Solanaceae; Golz et al. 2001). Deletion-type pollen-part SC mutant is also not known in Maloideae. In contrast, in *Prunus,* the mutation, even complete deletion, of *SFB* is not lethal to pollen, but, instead, confers pollen-part SC (Hauck et al. 2006a; Sonneveld et al. 2005; Tsukamoto et al. 2006; Ushijima et al. 2004). The difference of pollen *S* function between Prunoideae and Maloideae is consistent with the difference in the features of the pollen *S* (candidate) of them, i.e., the *SFB* of Prunoideae is single copy in each *S* haplotype, while the *SFBB/FBX*s of Maloideae are multiple, and more homologous to *SLFL* than to *SFB* of Prunoideae (Sassa et al. 2010).

Given that the *SFBB/FBX* genes show *S* haplotype-specific polymorphisms, linkage to the *S* locus and pollen-specific expression, they are candidates for pollen *S* of Maloideae. In the 317 kb *S* locus region of apple, no other good candidate was identified (Sassa et al. 2007). However, it seems unlikely that all the *SFBB/FBX* genes, more than ten in an *S* haplotype, are involved in pollen *S* specificity. The tentative nomenclature of *SFBB/FBX* genes should be revised in future based on the function of each member.

It has been postulated that pollen *S* protein degrades or inhibits the non-self S-RNases, possibly through ubiquitination, with leaving self S-RNase unaffected (Hua et al. 2008). In this scenario, heteroallelic pollen loses SI, because both two S-RNases are non-self for one of two pollen *S* proteins and thus are degraded/inhibited. If one or some of the *SFBB/FBX* genes is/are pollen *S* determinant(s), some mechanism may differentiate pollen *S* and non-*S* *SFBB/FBX* genes. Without such mechanism, non-*S* SFBB/FBX are likely to degrade/inhibit S-RNases irrespective of their *S* haplotypes, leading to SC. Pollen *S* SFBB/FBX proteins only are possible to be accumulated in pollen. It is also possible that pollen *S* SFBB/FBX but non-*S* SFBB/FBX can interact with S-RNase, although we detected no sequence motif that characterizes probable pollen *S* proteins among the SFBB/FBX.

Among the three families with the S-RNase based-GSI, Rosaceae is distantly related to the other two families Solanaceae and Plantaginaceae; the former belongs to subclass Rosidae while the latter two are in Asteridae. In addition, in Rosaceae, pollen-part SI function seems to differ among taxa, Maloideae and Prunoideae. Functional characterization of pollen *S* genes of Maloideae and comparisons to those of other species will provide insight into the evolutionary history and divergence of the S-RNase based-GSI.

Chromosomal location of the apple *S* locus

In this study, we physically mapped the apple *S* locus to the subtelomeric region of a chromosome using FISH. This is consistent with previous genetic linkage analyses that mapped the *S* locus to the bottom of linkage group 17 of apple (Igarashi et al. 2008; Maliepaard et al. 1998). FISH analysis also revealed that the apple *S* locus is not located near the centromeric region. Suppression of recombination at the *S* locus region is required to maintain the pair of the pistil- *S* and pollen-*S* alleles, the *S* haplotype, for SI function (de Nettancourt 2001; Franklin-Tong 2008). In Solanaceae, pericentromeric localization of the *S* locus has been considered to be an important factor in the suppression of recombination (Entani et al. 1999), while in *Brassica* and *Ipomoea*, the *S* loci were mapped to the subtelomeric regions and were not close to the centromeres (Iwano et al. 1998; Suzuki et al. 2004). In *Brassica* and *Ipomoea*, sequence heterogeneity among *S* haplotypes has been implicated for the suppression of recombination (Casselman et al. 2000; Iwano et al. 1998; Rahman et al. 2007; Suzuki et al. 2004). Given that the distance between *S-RNase* and *SFB* of *Prunus* is 380–40 kb (Ikeda et al. 2005), if one or more of the newly identified *MdFBX*s is involved in pollen *S* specificity, the physical size of the apple *S* locus would be remarkably larger than that of *Prunus*, because the *MdFBX*s are located outside the previously analyzed 317 kb *S* locus BAC contig (Sassa et al. 2007). A large-scale comparative sequence analysis of different apple *S* haplotypes would be useful to estimate the extent of the physical size and elucidate mechanism of recombination suppression of the *S* locus.

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