Journal of Experimental Botany, Vol. 62, No. 6, pp. 1887–1902, 2011
doi:10.1093/jxb/erq381 Advance Access publication 13 December, 2010
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Related polymorphic F-box protein genes between haplotypes clustering in the BAC contig sequences around the *S-RNase* of Japanese pear

Kazuma Okada[1,†], Nozomi Tonaka[1], Tomio Taguchi[1], Takehiko Ichikawa[1], Yutaka Sawamura[2], Tetsu Nakanishi[1] and Takeshi Takasaki-Yasuda[1,*]

[1] Graduate School of Agricultural Science, Kobe University, Kobe, Hyogo 657-8501, Japan
[2] National Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan

[†] Present address: National Institute of Fruit Tree Science, Morioka, Iwate 020-0123, Japan.
* To whom correspondence should be addressed. E-mail: taka@kobe-u.ac.jp

Received 20 March 2010; Revised 28 September 2010; Accepted 2 November 2010

Abstract

Most fruit trees in the Rosaceae exhibit self-incompatibility, which is controlled by the pistil *S* gene, encoding a ribonuclease (S-RNase), and the pollen *S* gene at the *S*-locus. The pollen *S* in *Prunus* is an F-box protein gene (*SLF*/*SFB*) located near the *S-RNase*, but it has not been identified in *Pyrus* and *Malus*. In the Japanese pear, various F-box protein genes (*PpSFBB*$^{-\alpha-\gamma}$) linked to the *S-RNase* are proposed as the pollen *S* candidate. Two bacterial artificial chromosome (BAC) contigs around the *S-RNase* genes of Japanese pear were constructed, and 649 kb around *S*$_4$*-RNase* and 378 kb around *S*$_2$*-RNase* were sequenced. Six and 10 pollen-specific F-box protein genes (designated as *PpSFBB*$^{4\text{-u1–u4, 4-d1–d2}}$ and *PpSFBB*$^{2\text{-u1–u5, 2-d1–d5}}$, respectively) were found, but *PpSFBB*$^{4-\alpha-\gamma}$ and *PpSFBB*$^{2-\gamma}$ were absent. The *PpSFBB*^{4} genes showed 66.2–93.1% amino acid identity with the *PpSFBB*^{2} genes, which indicated clustering of related polymorphic F-box protein genes between haplotypes near the *S-RNase* of the Japanese pear. Phylogenetic analysis classified 36 F-box protein genes of *Pyrus* and *Malus* into two major groups (I and II), and also generated gene pairs of *PpSFBB* genes and *PpSFBB*/*Malus* F-box protein genes. Group I consisted of gene pairs with 76.3–94.9% identity, while group II consisted of gene pairs with higher identities (>92%) than group I. This grouping suggests that less polymorphic *PpSFBB* genes in group II are non-*S* pollen genes and that the pollen *S* candidates are included in the group I *PpSFBB* genes.

Key words: BAC contig, F-box protein, pollen *S* gene, *Pyrus pyrifolia*, self-incompatibility, *S*-locus, *S-RNase*.

Introduction

Self-incompatibility (SI) is a genetic system that prevents self-fertilization in flowering plants by the recognition and rejection of self-pollen (de Nettancourt, 2001). In the Rosaceae, Solanaceae, and Plantaginaceae families, SI is classified as gametophytic SI (GSI), and is controlled by a single *S*-locus with multiple *S*-haplotypes. Each *S*-haplotype contains two genetically linked genes, the pistil *S* gene and the pollen *S* gene, which determine the *S*-haplotype specificity of the pistil and pollen, respectively (McCubbin and Kao, 2000). The pistil *S* encodes a ribonuclease known as S-RNase (McClure *et al.*, 1989; Ishimizu *et al.*, 1996; Xue *et al.*, 1996). The RNase activity of S-RNases is essential for rejection of self-pollen, and the degradation of rRNA by S-RNases inside the self-pollen tube results in inhibition of pollen growth (McClure *et al.*, 1990; Huang *et al.*, 1994). Thus, it is thought that the self S-RNase inhibits growth of the self-pollen tube via degradation of pollen rRNAs. On the other hand, the identity and function of the pollen *S* remained unknown for a long time. Recently, F-box protein genes were identified as the pollen *S* genes by

Abbreviations: GSI, gametophytic self-incompatibility; PFGE, pulsed field gel electrophoresis; SC, self-compatible; *SFB*, *S* haplotype-specific F-box protein; *SFBB*, *S* locus F-box brothers; SI, self-incompatibility; *SLF*, *S* locus F-box.

sequence analyses of cosmid and bacterial artificial chromosome (BAC) contigs around *S-RNase* in *Prunus* species of the Rosaceae, in *Petunia inflata* of the Solanaceae, and in *Antirrhinum hispanicum* of the Plantaginaceae. These F-box protein genes were termed *SLF* (*S*-locus F-box) or *SFB* (*S*-haplotype-specific F-box protein) (Lai *et al.*, 2002; Entani *et al.*, 2003; Ushijima *et al.*, 2003; Sijacic *et al.*, 2004). Transformation experiments in *P. inflata* and analyses of pollen-part self-compatible (SC) mutants in *Prunus* species provided evidence that *SLF*/*SFB* genes are the pollen *S* genes (Sijacic *et al.*, 2004; Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005; Hauck *et al.*, 2006; Tsukamoto *et al.*, 2006; Vilanova *et al.*, 2006). Generally, F-box proteins function as one of the four major subunits (CUL1, SKP1, RBX1, and F-box) that make up the SCF complex, which regulates protein stability through the ubiquitin–proteasome system (Lechner *et al.*, 2006). The model for S-RNase degradation proposes that the non-self-interaction between S-RNase and SLF/SFB leads to S-RNase ubiquitylation and degradation by the 26S proteasome (McClure and Franklin-Tong, 2006).

In Rosaceae, the pollen *S* has been identified only in *Prunus* (almond, apricots, and cherry), but not in *Pyrus* (pear) and *Malus* (apple). The Rosaceae comprises three subfamilies: Rosoideae, Dryadoideae, and Spiraeoideae. *Prunus*, *Pyrus*, and *Malus* are all included in Spiraeoideae (Potter *et al.*, 2007). Therefore, it is likely that the pollen *S* genes in *Pyrus* and *Malus* are also F-box protein genes. Recently, *S*-locus-linked and pollen-specific polymorphic F-box protein genes were isolated from apple (*Malus*×*domestica*) and Japanese pear (*Pyrus pyrifolia*), and these have been proposed as good candidates for the pollen *S* genes. Cheng *et al.* (2006) cloned two *S*-locus-linked F-box protein genes ($MdSLF_1$ and $MdSLF_2$) from apple by reverse transcription-PCR (RT-PCR) with degenerate primers designed from the conserved *SLF*/*SFB* sequences. Sassa *et al.* (2007) found several pollen-specific polymorphic F-box protein genes termed *SFBB* (*S* locus F-box brothers) in BAC contig sequences around apple *S-RNase* genes. These *SFBB* genes include $MdSFBB^{3\text{-}\alpha}$ and $MdSFBB^{3\text{-}\beta}$ around S_3-*RNase*, and $MdSFBB^{9\text{-}\alpha}$ and $MdSFBB^{9\text{-}\beta}$ around S_9-*RNase*. Using RT-PCR, they also cloned various *PpSFBB* genes ($PpSFBB^{\text{-}\alpha}$, $PpSFBB^{\text{-}\beta}$, and $PpSFBB^{\text{-}\gamma}$) that are linked to *S-RNase* genes of the Japanese pear; $PpSFBB^{4\text{-}\alpha}$, $PpSFBB^{4\text{-}\beta}$, and $PpSFBB^{4\text{-}\gamma}$ are linked to S_4-*RNase*, and $PpSFBB^{5\text{-}\alpha}$, $PpSFBB^{5\text{-}\beta}$, and $PpSFBB^{5\text{-}\gamma}$ are linked to S_5-*RNase*. $PpSFBB^{\text{-}\gamma}$ genes that are linked to another eight *S-RNase* genes have been cloned. They show high amino acid sequence identities (97.5–99.7%) among the 10 *S*-haplotypes (Kakui *et al.*, 2007). However, it is not clear whether *PpSFBB* genes are located near the *S-RNase*, like *MdSFBB* genes, or whether they are the pollen *S* genes. To identify the pollen *S* genes in the Japanese pear, a previously constructed BAC library from an S_4 homozygote was used and a BAC contig of ~570 kb around S_4-*RNase* was assembled. Sequence analysis of the 240 kb spanning 51 kb upstream to 189 kb downstream of S_4-*RNase* revealed a pollen-specific F-box protein gene (S_4F-*box0*; S_4-haplotype F-box protein gene) that differed from $PpSFBB^{4\text{-}\alpha\text{–}\gamma}$. S_4F-*box0* is located 127 kb downstream of S_4-*RNase* (Okada *et al.*, 2008). The SC cultivar 'Osa Nijisseiki' ($S_2S_4^{sm}$) is a natural mutant derived from 'Nijisseiki' (S_2S_4). The S_4-haplotype of 'Osa Nijisseiki' lacks the pistil *S* function but retains the pollen *S* function, and is termed the S_4^{sm}-haplotype, where 'sm' stands for 'stylar-part mutant' (Sato, 1993). The S_4^{sm}-haplotype has a 236 kb deletion, which includes S_4-*RNase* and S_4F-*box0*, suggesting that the pollen S_4 allele is located outside of the region spanning 48 kb upstream to 188 kb downstream of S_4-*RNase*—that is, outside the region that is deleted in the S_4^{sm}-haplotype (Okada *et al.*, 2008).

In this study, the sequence outside of the deleted region in S_4^{sm} was analysed, and the 649 kb region from 290 kb upstream to 359 kb downstream of S_4-*RNase* was determined; six $PpSFBB^4$ genes were found. To evaluate the *S*-haplotype polymorphism of $PpSFBB^4$ genes, a BAC library was constructed from the Japanese pear cultivar 'Choujuuro' (S_2S_3) to assemble a BAC contig around S_2-*RNase*. A 378 kb region from 166 kb upstream to 212 kb downstream of S_2-*RNase* was sequenced, and 10 $PpSFBB^2$ genes were found. Relationships among 36 F-box protein genes of *Pyrus* and *Malus* were analysed by comparing their amino acid sequences and by phylogenetic clustering.

Materials and methods

Plant materials

One cultivar and three *S* homozygotes of the Japanese pear were used: 'Choujuuro' (S_2S_3), and S_2, S_3, and S_4 homozygotes. The S_2 and S_3 homozygotes were selected from bud-selfed progeny of 'Choujuuro' (S_2S_3) (Terai *et al.*, 1999). The S_4 homozygote was segregated from bud-selfed progeny of 'Nijisseiki' (S_2S_4) (Okada *et al.*, 2008). The leaves, mature pollen, and pistils were frozen in liquid nitrogen, and stored at –80 °C until use.

Construction and characterization of an S_2S_3 BAC library

An S_2S_3 BAC library was constructed and characterized according to the method of Okada *et al.* (2008). High molecular weight DNA was isolated from leaf tissue (3 g) of 'Choujuuro' (S_2S_3), partially digested with *Hin*dIII, and size-selected twice by pulsed field gel electrophoresis (PFGE). In the first size selection, an agarose slice containing DNA fragments of 60–210 kb was excised and embedded into a new 1% SeaPlaque GTG agarose gel (Cambrex, http://www.cambrex.com/). In the second size selection, two size fractions (145–185 kb and 185–205 kb) were recovered by digestion of agarose slices with β-agarase I. DNA from each fraction was separately ligated into *Hin*dIII-digested CopyControl pCC1BAC Cloning-Ready vector (EPICENTRE, http://www.epibio.com/) and transformed into *Escherichia coli* strain TransforMax EPI300 (EPICENTRE). Equal numbers of transformed cells were picked from each fraction and a total of 61 440 colonies were pooled in 64 individual 96-well plates with 12 columns and eight rows (10 colonies per well) and stored at –80 °C. The BAC plasmid was extracted from the randomly chosen BAC clones by the standard alkaline lysis method, digested with *Not*I, and separated by PFGE. Insert size was estimated by comparison with a PFGE lambda ladder (New England Biolabs, http://www.neb.com/).

Chromosome walking

Chromosome walking in the region around *S-RNase* was performed by PCR screening of the S_2S_3 BAC library and the previously constructed S_4S_4 BAC library (Okada *et al.*, 2008). The PCR screening was performed in three consecutive steps as described by Okada *et al.* (2008). Chromosome walking around the *S_2-RNase* was initiated by PCR screening of the S_2S_3 BAC library with an *S-RNase*-specific primer pair, 'FTQQYQ' and 'anti-IIWPNV' (Ishimizu *et al.*, 1999).

BAC plasmids were isolated from positive BAC clones using the Plasmid Midi Kit (Qiagen, http://www1.qiagen.com/). Both ends of the BACs (~600 bp) of the positive clones were sequenced using T7 and RP vector primers, and a primer pair was designed from each BAC-end sequence (Supplementary Table S1 available at *JXB* online). For chromosome walking, non-repetitive primer pairs were selected from the BAC-end primer pairs located at the outer ends of the contig by PCR amplification of plate pool templates, which were prepared by mixing all 960 BAC clones in each plate. Furthermore, S_2-specific primer pairs were identified from among the non-repetitive primer pairs by PCR, using genomic DNA of the S_2 and S_3 homozygotes as templates. These S_2-specific primer pairs were used for PCR screening of the BAC library (Supplementary Table S1). For PCR, genomic DNA was isolated from leaves of the S_2 and S_3 homozygotes by the modified cetyltrimethylammonium bromide (CTAB) method (Castillo *et al.*, 2001).

To estimate insert sizes and compare restriction patterns, BAC plasmids were digested with *Not*I and subjected to PFGE. Based on overlapping of the BAC clones, their insert sizes, and restriction patterns, the physical distance was calculated to construct a BAC contig.

BAC subcloning

The plasmids of BAC clones were completely digested with *Hin*dIII or *Eco*RI, separated on a 0.7% agarose gel, and purified from the gels using GENECLEAN Kit III (Qbiogene, http://www.qbiogene.com/). Each fragment was ligated into pBluescriptII SK (+) and transformed into *E. coli* strain TOP10F' (Invitrogen, http://www.invitrogen.com/). Inserts from subclones that were smaller than 7 kb were sequenced by primer walking, and those that were larger than 7 kb were sequenced after subcloning using other restriction enzymes. A primer was designed from each insert-end sequence. Using these primers, the regions outside of the subclones in the BAC plasmids were sequenced. The sequences from subclones and the outside sequences were assembled to construct contigs for each BAC clone. Gap regions, for which no sequence data were obtained, were amplified from BAC plasmids by PCR with the Expand High Fidelity PCR system (Roche Diagnostics, http://www.roche-diagnostics.jp/), and directly sequenced.

Nucleotide and amino acid sequence analysis

Nucleotide sequences were determined with the BigDye Terminator v3.1 Cycle Sequencing Kit using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, http://www3.appliedbiosystems.com/). The sequence data were analysed using GENETYX-MAC Ver. 13 and ATGC Ver. 4 software packages (Genetyx, http://www.sdc.co.jp/genetyx/). Protein-coding sequences were predicted using the GENSCAN program (Burge and Karlin, 1997). Homology searches were carried out using the BLASTX program (Altschul *et al.*, 1997). Deduced amino acid sequences were analysed by Pfam (http://pfam.janelia.org/) to search for protein motifs. Amino acid sequences were aligned using ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html) and manually optimized. A phylogenetic tree was constructed by the Neighbor–Joining method (Saitou and Nei, 1987). A Harr plot analysis was performed using GENETYX-MAC Ver. 13 software.

RT-PCR

Total RNAs extracted from pollen, pistils, and leaves were subjected to first-strand cDNA synthesis using ReverTra Ace α (TOYOBO, http://www.toyobo.co.jp/). Using the Expand High Fidelity PCR system (Roche Diagnostics), PCR was then carried out with gene-specific primer pairs (Supplementary Table S2 at *JXB* online). PCR products were separated by electrophoresis on 1.5% or 0.8% agarose gels and visualized by ethidium bromide staining. The PCR products were purified from the gels, and sequenced to confirm gene specificity.

Sequence data

The 649 kb and 378 kb sequences around S_4- and *S_2-RNase* genes have been deposited in the EMBL/GenBank Data Libraries under accession nos AB545981 and AB545982, respectively.

Results

Sequence analysis of 649 kb around the S_4-RNase *gene*

Previously, an S_4 BAC contig spanning 202 kb upstream (18F9 T7-end) to 359 kb downstream (33G4 T7-end) of *S_4-RNase* was constructed. In addition, the complete sequences of two BAC clones (17C7 and 5D3) and the partial sequences of two other BAC clones (32D11 and 4D9) were analysed to determine a 240 001 bp sequence spanning 51 kb upstream to 189 kb downstream of *S_4-RNase* (Okada *et al.*, 2008). In this study, 32D11 and 4D9 were sequenced in their entirety. To extend the S_4 BAC contig upstream, chromosome walking was resumed using a non-repetitive BAC-end primer pair (33H11-T7). PCR screening of the S_4S_4 BAC library yielded five BAC clones: 12B8, 15C1, 31C7, 31F1, and 36B6. As a result, chromosome walking from *S_4-RNase* produced a set of overlapping BAC clones (36B6, 18F9, 32D11, 17C7, 5D3, 4D9, and 33G4) covering ~649 kb, spanning 290 kb upstream to 359 kb downstream of *S_4-RNase* (Fig. 1A). Three BAC clones (36B6, 18F9, and 33G4) were subcloned and completely sequenced. The sequence assembly of the seven BAC clones (36B6, 18F9, 32D11, 17C7, 5D3, 4D9, and 33G4) yielded a 648 516 bp sequence spanning 290 kb upstream to 359 kb downstream of the *S_4-RNase*.

Analysis using GENSCAN software predicted 89 open reading frames (ORFs) in the S_4 BAC 649 kb contig sequence (Fig. 2A). Among the 89 ORFs, 34 (ORF33–ORF66) were included in the 236 kb deleted region of the S_4^{sm}-haplotype that spans 48 kb upstream to 188 kb downstream of *S_4-RNase* (Fig. 2A; Okada *et al.*, 2008). The other 55 ORFs (ORF1–ORF32 and ORF67–ORF89) were located outside the deleted region. A BLASTX search of the 89 ORFs yielded 61 ORFs with significant similarity (E-value <e-4) to sequences of known proteins in the database (Table 1). Of the 89 ORFs, 40 showed similarity to a (retro) transposon, and 10 were similar to a hypothetical or predicted protein. ORF14 was similar to a zinc knuckle family protein, ORF43 to a zinc finger, ORF50 to a chromosome-associated kinesin KIF4A, and ORF62 to an unknown protein. ORF42 and ORF54 corresponded to

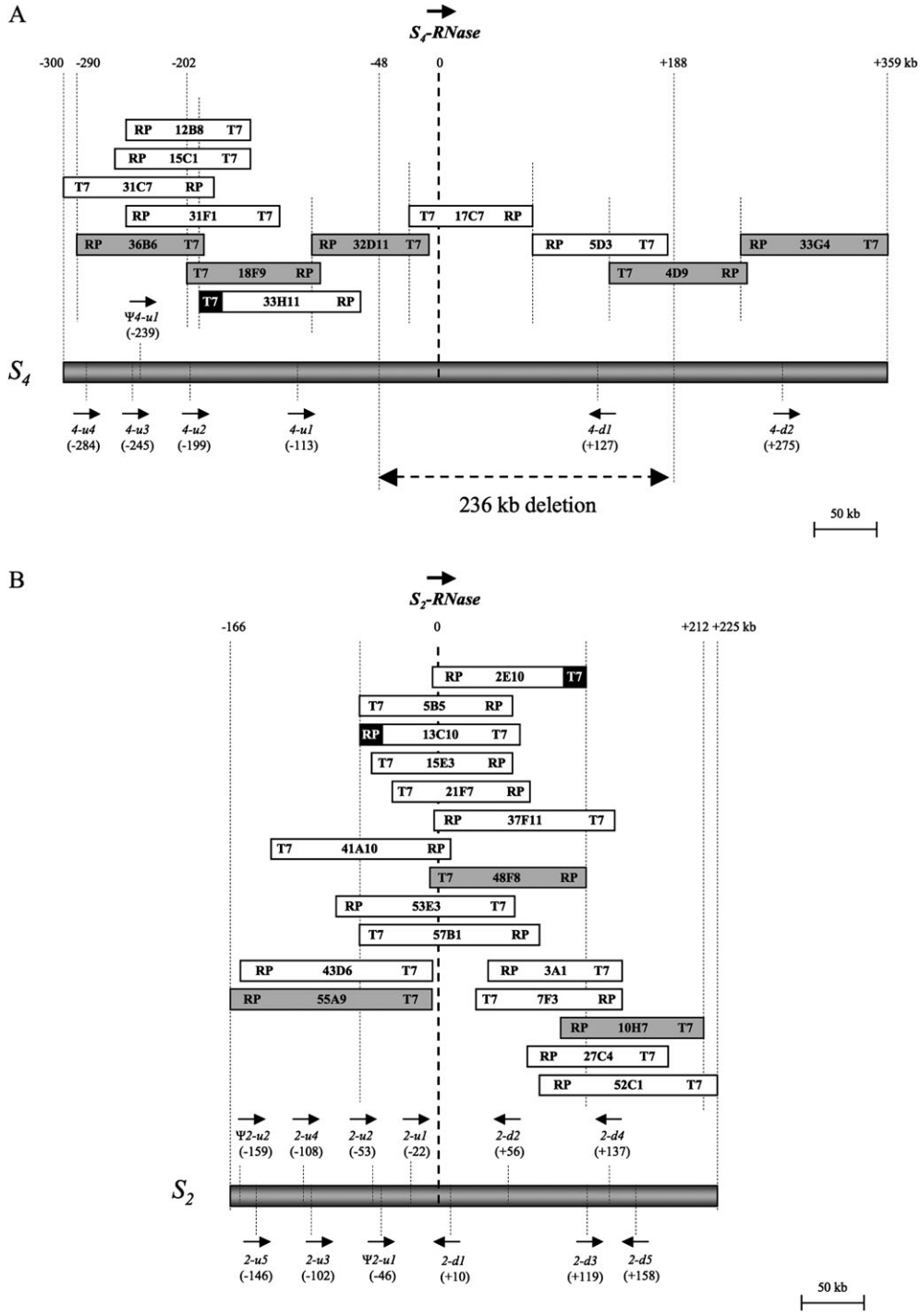


Fig. 1. Construction of BAC contigs, schematic genomic structures, and locations of (pseudo) F-box protein genes around *S_4-RNase* (A) and *S_2-RNase* (B) of the Japanese pear. Names, T7-, and RP-ends of BAC clones are shown in boxes. Black ends represent BAC-ends used for chromosome walking. Hatched boxes indicate BAC clones chosen for complete sequencing. Schematic genomic structures of S_4- and S_2-haplotypes are shown below the BAC contigs. The directions of transcription of *S-RNase* and *PpSFBB* genes are represented by arrows. Physical distances from *S-RNase* are indicated in parentheses. A double-headed arrow indicates the 236 kb deleted region in the S_4^{sm}-haplotype.

S_4-RNase and *S_4F-box0*, respectively. Five ORFs (ORF3, ORF9, ORF15, ORF25, and ORF79) were located outside the deleted region, and showed similarity to *MdSFBBs* and *PpSFBB*$^{9\text{-}\gamma}$. Using GENETYX-MAC Ver. 13 software, the predicted ORFs were reanalysed to determine the precise ORFs from the start (ATG) to the stop codon. ORF3, ORF9, ORF15, ORF25, and ORF79 encoded 410, 390, 403, 394, and 393 amino acid residues, respectively. A Pfam motif search predicted that these five proteins had an F-box domain at the N-terminus and an FBA_1 domain in the

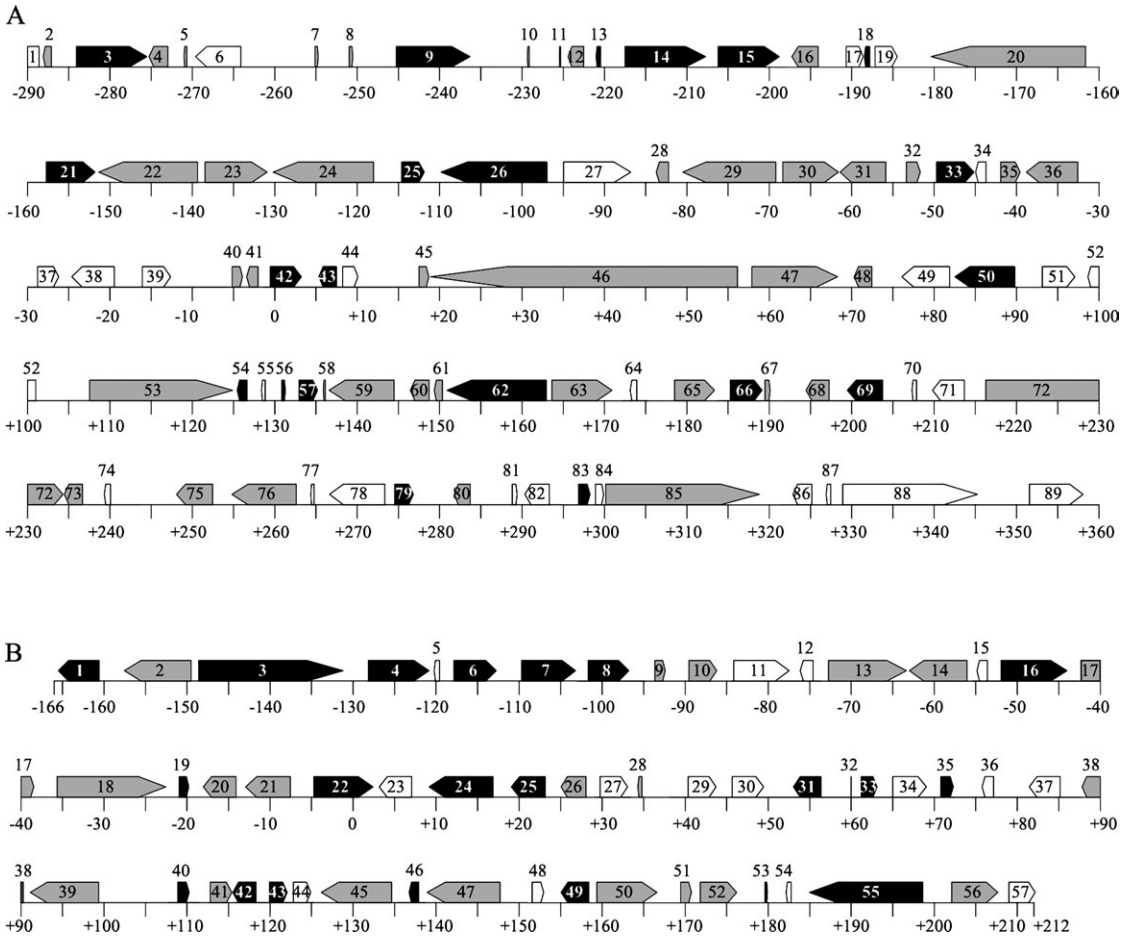


Fig. 2. ORF maps of the region around S_4-*RNase* (A) and S_2-*RNase* (B). Arrowheads indicate the location and transcriptional direction of genes predicted by GENSCAN software. Open arrowheads indicate genes showing no significant homology to proteins in databases. Grey arrowheads represent transposable elements. Black arrowheads indicate non-transposon-like genes. The 649 kb and 378 kb sequences around S_4-*RNase* and S_2-*RNase* have been deposited with the EMBL/GenBank Data Libraries under accession nos AB545981 and AB545982, respectively.

centre (Fig. 3). These ORFs showed pairwise deduced amino acid sequence identities ranging from 62.9% to 94.4% when compared with reported *PpSFBB*, *MdSFBB*, and *MdSLF* genes (Supplementary Table S3 at *JXB* online). They represent F-box protein genes that differ from $PpSFBB^{4\text{-}\alpha\text{–}\gamma}$, which are linked to S_4-*RNase* (Sassa *et al.*, 2007). Thus, these five ORFs were assigned as new $PpSFBB^4$ genes. ORF3, ORF9, ORF15, and ORF25 were located ~284, ~245, ~199, and ~113 kb upstream of S_4-*RNase*. ORF54 (S_4*F-box0*) and ORF79 were located ~127 kb and ~275 kb downstream of S_4-*RNase*. These $PpSFBB^4$ genes upstream and downstream of S_4-*RNase* were named $PpSFBB^{4\text{-u}}$ and $PpSFBB^{4\text{-d}}$, respectively, and lower case numbers were assigned to the $PpSFBB^{4\text{-u}}$ and $PpSFBB^{4\text{-d}}$ located close to S_4-*RNase*; therefore, ORF25, ORF15, ORF9, and ORF3 were designated as $PpSFBB^{4\text{-u1}}$, $PpSFBB^{4\text{-u2}}$, $PpSFBB^{4\text{-u3}}$, and $PpSFBB^{4\text{-u4}}$, and ORF54 (S_4*F-box0*) and ORF79 were (re)named as $PpSFBB^{4\text{-d1}}$ and $PpSFBB^{4\text{-d2}}$, respectively. These $PpSFBB^4$ genes around S_4-*RNase* shared the same transcriptional orientation, except for $PpSFBB^{4\text{-d1}}$ (Fig. 1A). Using ATGC Ver. 4 software, the S_4 649 kb BAC contig sequence was searched for *SFBB*-like sequences. The analysis revealed a pseudogene ($\Psi PpSFBB^{4\text{-u1}}$) encoding a truncated F-box protein at ~239 kb upstream of S_4-*RNase* (Fig. 1A). $PpSFBB^{4\text{-u1–u4}}$ and $PpSFBB^{4\text{-d1–d2}}$ shared 67.2–86.2% amino acid sequence identities with each other (Table 3).

To examine expression of $PpSFBB^{4\text{-u1–u4}}$ and $PpSFBB^{4\text{-d1–d2}}$, total RNA was extracted from pollen, pistils, and leaves of the S_4 homozygote. RT-PCR analyses were conducted using gene-specific primer pairs (Supplementary Table S2 at *JXB* online). $PpSFBB^{4\text{-u1–u4}}$ and $PpSFBB^{4\text{-d1–d2}}$ were all specifically expressed in pollen, but not in pistils or leaves (Supplementary Fig. S1A). The $PpSFBB^{4\text{-d2}}$-specific primer pair yielded fragments of 1373 bp and 1142 bp, which both were derived from the $PpSFBB^{4\text{-d2}}$ transcript, because the forward primer annealed to the 5' untranslated region (UTR) and the coding region of $PpSFBB^{4\text{-d2}}$ (Supplementary Table S2, Fig. S1A). Thus, in the 649 kb sequence around S_4-*RNase* there were six F-box protein genes ($PpSFBB^{4\text{-u1–u4}}$ and $PpSFBB^{4\text{-d1–d2}}$) with pollen-specific expression. The three *PpSFBB* genes previously shown to be linked to the S_4-*RNase*, $PpSFBB^{4\text{-}\alpha\text{–}\gamma}$, were not within the sequenced region.

Table 1. *Open reading frames (ORFs) predicted by GENSCAN in the 649 kb region around* S_4-RNase

ORFs		Homologous protein	Species	Amino acid identity	Score (bits)	E-value[a]	Accession no.
ORF1		None					
ORF2		Transposon protein	*Oryza sativa*	64/122 (52%)	134	9e-30	DP000011
ORF3	(*SFBB*$^{4\text{-u4}}$)	PpSFBB$^{9\text{-}\gamma}$	*Pyrus pyrifolia*	291/378 (76%)	566	4e-159	AB297939
ORF4		Putative retroelement pol polyprotein	*Arabidopsis thaliana*	24/36 (66%)	50.8	4e-05	AC006920
ORF5		GAG-POL precursor	*Vitis vinifera*	34/124 (27%)	61.2	3e-08	AB111100
ORF6		None					
ORF7		Retrotransposon protein	*Oryza sativa*	45/107 (42%)	79.7	9e-14	DP000009
ORF8		Retrotransposon protein	*Oryza sativa*	45/158 (28%)	82.4	1e-14	DP000009
ORF9	(*SFBB*$^{4\text{-u3}}$)	MdSFBB$^{9\text{-}\beta}$	*Malus*×*domestica*	350/390 (89%)	726	0.0	AB270792
ORF10		Retrotransposon protein	*Oryza sativa*	31/78 (39%)	54.3	6e-08	DP000011
ORF11		None					
ORF12		Retrotransposon protein	*Oryza sativa*	36/82 (43%)	81.6	6e-14	DP000009
ORF13		Hypothetical protein	*Vitis vinifera*	59/181 (32%)	71.6	3e-11	AM472051
ORF14		Zinc knuckle family protein	*Oryza sativa*	60/182 (32%)	100	4e-19	DP000010
ORF15	(*SFBB*$^{4\text{-u2}}$)	MdSFBB$^{9\text{-}\alpha}$	*Malus*×*domestica*	300/315 (95%)	590	4e-166	AB270792
ORF16		Retrotransposon protein	*Oryza sativa*	71/165 (43%)	124	7e-27	DP000009
ORF17		None					
ORF18		Predicted protein	*Populus trichocarpa*	44/134 (32%)	69.7	2e-10	DS017968
ORF19		None					
ORF20		Retrotransposon protein	*Oryza sativa*	31/81 (38%)	60.1	1e-09	DP000086
ORF21		Hypothetical protein	*Vitis vinifera*	43/132 (32%)	70.5	1e-10	AM426737
ORF22		Retrotransposon protein	*Beta vulgaris*	61/173 (35%)	82.0	3e-13	EF101866
ORF23		Retrotransposon gag protein	*Asparagus officinalis*	418/767 (54%)	772	0.0	AC183435
ORF24		Retrotransposon gag protein	*Oryza sativa*	487/1032 (47%)	921	0.0	AC120534
ORF25	(*SFBB*$^{4\text{-u1}}$)	MdSFBB$^{3\text{-}\beta}$	*Malus*×*domestica*	306/394 (77%)	628	4e-178	AB270796
ORF26		Hypothetical protein	*Vitis vinifera*	28/42 (66%)	64.3	4e-08	AM455744
ORF27		None					
ORF28		Reverse transcriptase	*Vigna radiata*	23/37 (62%)	48.5	2e-04	AY684634
ORF29		Retrotransposon protein	*Oryza sativa*	521/962 (54%)	1028	0.0	DP000011
ORF30		Retrotransposon gag protein	*Asparagus officinalis*	48/129 (37%)	91.3	2e-16	AC183436
ORF31		Retrotransposon gag protein	*Asparagus officinalis*	827/1636 (50%)	1568	0.0	AC183435
ORF32		Integrase	*Populus trichocarpa*	48/149 (32%)	84.0	3e-14	DQ536160
ORF33		Predicted protein	*Populus trichocarpa*	34/92 (36%)	62.4	3e-08	EQ134071
ORF34		None					
ORF35		Retrotransposon gag protein	*Asparagus officinalis*	95/194 (48%)	172	4e-41	AC183435
ORF36		Retrotransposon gag protein	*Asparagus officinalis*	117/405 (28%)	141	2e-31	AC183435
ORF37		None					
ORF38		None					
ORF39		None					
ORF40		Transposon protein Pong subclass	*Zea mays*	31/120 (25%)	60.1	3e-07	EU964924
ORF41		Transposon protein Pong subclass	*Zea mays*	185/380 (48%)	355	8e-96	EU962682
ORF42	(*S_4-RNase*)	S_4-RNase	*Pyrus pyrifolia*	49/50 (98%)	115	2e-24	AB014072
ORF43		Zinc finger	*Medicago truncatula*	23/63 (36%)	47.8	6e-04	AC148290
ORF44		None					
ORF45		Retrotransposon gag protein	*Asparagus officinalis*	65/291 (22%)	59.7	5e-07	AC183435
ORF46		Retroelement pol polyprotein-like	*Arabidopsis thaliana*	667/1331 (50%)	1272	0.0	AB024037
ORF47		Retrotransposon gag protein	*Asparagus officinalis*	793/1644 (48%)	1496	0.0	AC183435
ORF48		Retrotransposon protein	*Oryza sativa*	352/728 (48%)	673	0.0	DP000011
ORF49		None					
ORF50		Chromosome-associated kinesin KIF4A	*Ricinus communis*	37/96 (38%)	60.5	2e-07	EQ974117
ORF51		None					
ORF52		None					
ORF53		Retrotransposon protein	*Beta vulgaris*	101/201 (50%)	191	7e-46	EF101866
ORF54	(*SFBB*$^{4\text{-d1}}$)	S_4F-box0	*Pyrus pyrifolia*	400/400 (100%)	834	0.0	AB308360
ORF55		None					
ORF56		Hypothetical protein	*Vitis vinifera*	45/154 (29%)	72.0	2e-11	AM429787
ORF57		Hypothetical protein	*Vitis vinifera*	50/135 (37%)	81.3	2e-13	AM467140

Table 1. *Continued*

ORFs		Homologous protein	Species	Amino acid identity	Score (bits)	E-value[a]	Accession no.
ORF58		RNase H family protein	*Asparagus officinalis*	35/63 (55%)	78.6	2e-18	AC183436
ORF59		Retrotransposon protein	*Oryza sativa*	67/121 (55%)	137	1e-30	DP000009
ORF60		Retrotransposon protein	*Oryza sativa*	127/297 (42%)	234	2e-59	DP000009
ORF61		Retrotransposon gag protein	*Asparagus officinalis*	37/129 (28%)	74.7	1e-11	AC183435
ORF62		Unknown protein	*Arabidopsis thaliana*	51/74 (68%)	102	6e-20	AK117191
ORF63		Retrotransposon gag protein	*Asparagus officinalis*	119/299 (39%)	195	3e-47	AC183435
ORF64		None					
ORF65		Retrotransposon protein	*Beta vulgaris*	35/44 (79%)	76.3	2e-18	EF101866
ORF66		Hypothetical protein	*Vitis vinifera*	39/110 (35%)	55.1	5e-06	AM489256
ORF67		RNase H family protein	*Asparagus officinalis*	58/121 (47%)	115	2e-24	AC183436
ORF68		Retrotransposon gag protein	*Asparagus officinalis*	229/692 (33%)	341	2e-91	AC183435
ORF69		Hypothetical protein	*Vitis vinifera*	68/227 (29%)	84.7	3e-14	AM451669
ORF70		None					
ORF71		None					
ORF72		Retrotransposon gag protein	*Asparagus officinalis*	714/1299 (54%)	1440	0.0	AC183435
ORF73		Retrotransposon gag protein	*Asparagus officinalis*	401/608 (65%)	821	0.0	AC183435
ORF74		None					
ORF75		Integrase	*Populus trichocarpa*	212/535 (39%)	357	2e-96	DQ536178
ORF76		Retrotransposon gag protein	*Asparagus officinalis*	167/506 (33%)	223	1e-55	AC183435
ORF77		None					
ORF78		None					
ORF79	(*SFBB*$^{4\text{-d2}}$)	MdSFBB$^{3\text{-}\beta}$	*Malus*×*domestica*	293/393 (74%)	561	4e-158	AB270796
ORF80		Retrotransposon protein	*Oryza sativa*	175/487 (35%)	225	9e-57	DP000009
ORF81		None					
ORF82		None					
ORF83		Hypothetical protein	*Vitis vinifera*	27/46 (58%)	56.2	1e-06	AM482339
ORF84		None					
ORF85		Retrotransposon protein	*Oryza sativa*	242/432 (56%)	471	4e-130	DP000009
ORF86		None					
ORF87		None					
ORF88		None					
ORF89		None					

[a] Significant similarity corresponds to an E-value <e $^{-4}$.

Construction of a BAC contig around the S_2-RNase *gene*

To analyse the sequence polymorphism of *PpSFBB*$^{4\text{-u1–u4}}$ and *PpSFBB*$^{4\text{-d1–d2}}$ in another haplotype, a BAC library was constructed from the Japanese pear cultivar 'Choujuuro' (S_2S_3). The BAC library consisted of two sublibraries derived from two DNA size fractions. One sublibrary, which was derived from the 145–185 kb size fraction, consisted of 30 720 clones with an average insert size of 111 kb. The other sublibrary, which was derived from the 185–205 kb size fraction, consisted of 30 720 clones with an average insert size of 127 kb. The average insert size of the whole BAC library was ~119 kb. The haploid genome size of pear is estimated to be 496–536 Mb (Arumuganathan and Earle, 1991). Therefore, the BAC library represented ~14-fold genome coverage, giving a >99% theoretical probability of recovering any single-copy DNA sequences in the genome.

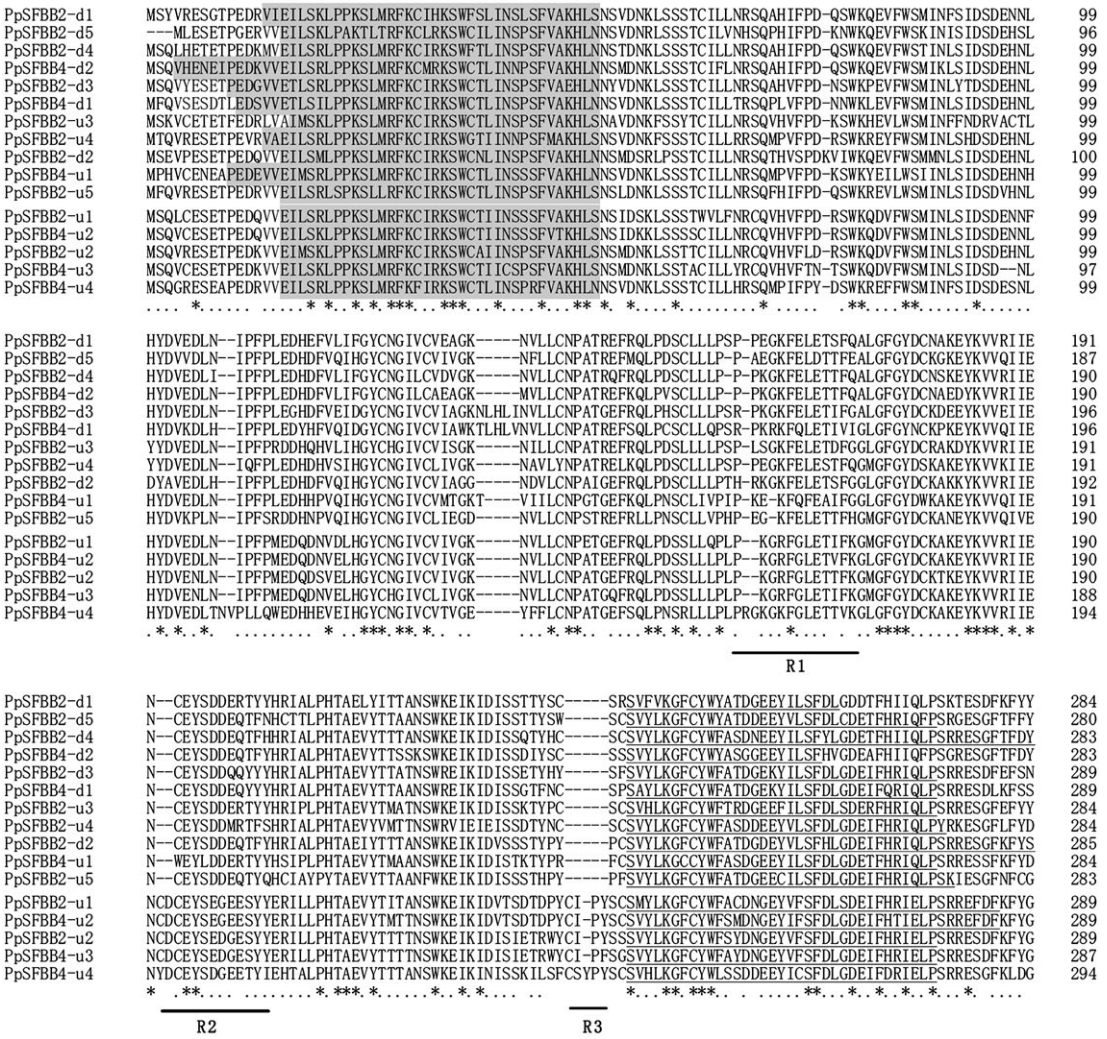
To construct a BAC contig around *S_2-RNase*, chromosome walking was initiated from *S_2-RNase*. PCR screening of the BAC library of 'Choujuuro' with an *S-RNase*-specific primer pair yielded 10 BAC clones containing *S_2-RNase*: 2E10, 5B5, 13C10, 15E3, 21F7, 37F11, 41A10, 48F8, 53E3, and 57B1. These BAC clones were aligned by PCR analysis with primer pairs designed from each BAC-end sequence, and a first contig was constructed based on the insert size and restriction pattern of the BAC plasmids (Fig. 1B).

For chromosome walking, two non-repetitive and S_2-haplotype specific primer pairs, 13C10-RP and 2E10-T7, were selected from the BAC-end primer pairs located at the outer ends of the first contig (Supplementary Table S1 at *JXB* online). PCR screening of the BAC library with 13C10-RP yielded two BAC clones (43D6 and 55A9) upstream of *S_2-RNase.* PCR screening of the BAC library with 2E10-T7 yielded five BAC clones (3A1, 7F3, 10H7, 27C4, and 52C1) downstream of *S_2-RNase*. Finally, chromosome walking from *S_2-RNase* yielded a total of 17 BAC clones. These were aligned to construct a BAC contig of ~391 kb spanning 166 kb upstream to 225 kb downstream of *S_2-RNase* (Fig. 1B).

Table 2. *Open reading frames (ORFs) predicted by GENSCAN in the 378 kb region around* S_2-RNase

ORFs		Homologous protein	Species	Amino acid identity	Score (bits)	E-value[a]	Accession no.
ORF1		Serine-threonine protein kinase	*Ricinus communis*	262/411 (63%)	506	2e-141	EQ974075
ORF2		Retrotransposon protein	*Oryza sativa*	189/479 (39%)	355	1e-95	DP000011
ORF3	(*SFBB*$^{2\text{-u5}}$)	S_2-locus F-box	*Malus*×*domestica*	280/312 (89%)	602	9e-170	DQ422811
ORF4		DNA glycosylase DEMETER	*Arabidopsis thaliana*	351/1051 (33%)	385	1e-110	DQ335243
ORF5		None					
ORF6		DNA glycosylase	*Populus trichocarpa*	196/291 (67%)	365	4e-99	CM000346
ORF7	(*SFBB*$^{2\text{-u4}}$)	PpSFBB$^{4\text{-}\beta}$	*Pyrus pyrifolia*	376/396 (94%)	752	0.0	AB270798
ORF8	(*SFBB*$^{2\text{-u3}}$)	S_1-locus F-box	*Malus*×*domestica*	369/394 (93%)	747	0.0	DQ422810
ORF9		Retrotransposon protein	*Oryza sativa*	78/248 (31%)	104	2e-20	DP000010
ORF10		Retrotransposon protein	*Oryza sativa*	132/267 (49%)	249	8e-64	DP000010
ORF11		None					
ORF12		None					
ORF13		Retrotransposon protein	*Oryza sativa*	38/82 (46%)	83.6	3e-14	DP000086
ORF14		GAG-POL precursor	*Vitis vinifera*	65/208 (31%)	105	2e-20	AB111100
ORF15		None					
ORF16	(*SFBB*$^{2\text{-u2}}$)	MdSFBB$^{9\text{-}\beta}$	*Malus*×*domestica*	369/391 (94%)	768	0.0	AB270792
ORF17		Retrotransposon protein	*Oryza sativa*	332/820 (40%)	570	4e-160	DP000011
ORF18		Retrotransposon protein	*Oryza sativa*	96/180 (53%)	184	5e-44	DP000011
ORF19	(*SFBB*$^{2\text{-u1}}$)	MdSFBB$^{9\text{-}\alpha}$	*Malus*×*domestica*	361/392 (92%)	694	0.0	AB270792
ORF20		Retrotransposon protein	*Oryza sativa*	269/693 (38%)	466	8e-129	DP000009
ORF21		Retrotransposon protein	*Beta vulgaris*	41/57 (71%)	89.0	9e-16	EF101866
ORF22	(*S_2-RNase*)	S_2-RNase	*Pyrus pyrifolia*	191/191 (100%)	410	5e-112	AB014073
ORF23		None					
ORF24	(*SFBB*$^{2\text{-d1}}$)	MdSFBB$^{3\text{-}\alpha}$	*Malus*×*domestica*	366/394 (92%)	735	0.0	AB270795
ORF25		Hypothetical protein	*Vitis vinifera*	22/36 (61%)	46.2	3e-09	AM426737
ORF26		Retrotransposon protein	*Oryza sativa*	34/68 (50%)	80.9	4e-13	DP000009
ORF27		None					
ORF28		Retrotransposon protein	*Beta vulgaris*	25/38 (65%)	54.3	4e-06	EF101866
ORF29		None					
ORF30		None					
ORF31	(*SFBB*$^{2\text{-d2}}$)	MdSFBB$^{3\text{-}\beta}$	*Malus*×*domestica*	304/394 (77%)	637	0.0	AB270796
ORF32		None					
ORF33		Hypothetical protein	*Vitis vinifera*	69/195 (35%)	88.2	9e-16	AM483001
ORF34		None					
ORF35		Hypothetical protein	*Vitis vinifera*	49/180 (27%)	60.8	2e-07	AM423348
ORF36		None					
ORF37		None					
ORF38		Retroelement pol polyprotein-like	*Arabidopsis thaliana*	151/243 (62%)	238	1e-60	AB024037
ORF39		Retrotransposon gag protein	*Asparagus officinalis*	72/152 (47%)	146	5e-33	AC183435
ORF40		TIR-NBS-LRR-type disease resistance protein	*Populus trichocarpa*	109/213 (51%)	204	3e-51	DQ513203
ORF41		LTR retrotransposon like protein	*Arabidopsis thaliana*	148/283 (52%)	283	4e-74	AL022140
ORF42		TIR-NBS-LRR-type disease resistance protein	*Populus trichocarpa*	66/90 (73%)	137	3e-31	DQ513203
ORF43	(*SFBB*$^{2\text{-d3}}$)	S_4F-box0	*Pyrus pyrifolia*	331/400 (82%)	681	0.0	AB308360
ORF44		None					
ORF45		Retrotransposon protein	*Oryza sativa*	145/328 (44%)	249	2e-63	DP000011
ORF46	(*SFBB*$^{2\text{-d4}}$)	MdSFBB$_{3\text{-}\beta}$	*Malus*×*domestica*	366/392 (93%)	772	0.0	AB270796
ORF47		Putative retroelement polyprotein	*Arabidopsis thaliana*	388/919 (42%)	652	0.0	AC018460
ORF48		None					
ORF49	(*SFBB*$^{2\text{-d5}}$)	MdSFBB$^{3\text{-}\beta}$	*Malus*×*domestica*	309/388 (79%)	647	0.0	AB270796
ORF50		Retrotransposon protein	*Oryza sativa*	178/526 (33%)	233	1e-58	DP000010
ORF51		Polyprotein 1	*Petunia vein clearing virus*	67/284 (23%)	66.2	7e-09	AY228106
ORF52		Retrotransposon protein	*Oryza sativa*	145/350 (41%)	255	2e-65	DP000011
ORF53		Cyclin-like F-box	*Medicago truncatula*	41/89 (46%)	88.2	2e-16	AC150889
ORF54		None					
ORF55		Hypothetical protein	*Prunus persica*	42/91 (46%)	92.8	6e-17	DQ863257
ORF56		Retrotransposon protein	*Beta vulgaris*	64/86 (74%)	139	7e-31	EF101866
ORF57		None					

[a] Significant similarity corresponds to an E-value <e $^{-4}$.


R1
R2
R3
R4
R5

Sequence analysis of 378 kb around the S_2-RNase gene

To identify the genes around *S_2-RNase*, three overlapping BAC clones, 55A9, 48F8, and 10H7, were subcloned and completely sequenced (Fig. 1B). Sequence assembly of the three BAC clones yielded a 378 419 bp sequence. Analysis using GENSCAN software predicted 57 ORFs in the 378 kb region (Fig. 2B). A BLASTX search of these ORFs yielded 41 ORFs with significant similarity (E-value <e-4) to sequences of known proteins in the database (Table 2). ORF22 corresponded to *S_2-RNase*. Among the 57 ORFs, 20 were similar to (retro) transposons and four were similar to a hypothetical protein. ORF1 was similar to a serine-threonine protein kinase, ORF4 to the DNA glycosylase DEMETER, ORF6 to a DNA glycosylase, ORF40 and ORF42 to TIR-NBS-LRR-type disease resistance proteins, and ORF53 to a cyclin-like F-box. Ten ORFs (ORF3, ORF7, ORF8, ORF16, ORF19, ORF24, ORF31, ORF43, ORF46, and ORF49) showed high sequence similarity to *MdSFBB* genes, *MdSLF* genes, *PpSFBB*$^{4\text{-}\beta}$, or *S_4F-box0*. Using GENETYX-MAC Ver. 13 software, the predicted ORFs were reanalysed to determine the precise ORFs from the start (ATG) to the stop codon. ORF3, ORF7, ORF8, ORF16, ORF19, ORF24, ORF31, ORF43, ORF46, and ORF49 encoded 393, 396, 394, 392, 392, 394, 395, 400, 393, and 390 amino acid residues, respectively. A Pfam motif search predicted that these proteins had an F-box domain at the N-terminus and an FBA_1 domain in the centre (Fig. 3). When compared with reported *PpSFBB*, *MdSFBB*, and *MdSLF* genes, these ORFs showed pairwise deduced amino acid sequence identities ranging from 62.0% to 94.9% (Supplementary Table S3 at *JXB* online). The F-box protein genes differed from *PpSFBB*$^{2\text{-}\gamma}$, which was reported to be linked to *S_2-RNase* (Kakui *et al.*, 2007). Thus, these ORFs were assigned as new *PpSFBB*^{2} genes. ORF3, ORF7, ORF8, ORF16, and ORF19 were located ~146, ~108, ~102, ~53, and ~22 kb, respectively, upstream of *S_2-RNase*. ORF24, ORF31, ORF43, ORF46, and ORF49 were located ~10, ~56, ~119, ~137, and ~158 kb, respectively, downstream of *S_2-RNase*. These new *PpSFBB*^{2} genes upstream and downstream of *S_2-RNase* were named *PpSFBB*$^{2\text{-u}}$ and *PpSFBB*$^{2\text{-d5}}$, respectively, and lower case numbers were assigned to the *PpSFBB*$^{2\text{-u}}$ and *PpSFBB*$^{2\text{-d}}$ located close to *S_2-RNase*. Therefore, ORF19, ORF16, ORF8, ORF7, and ORF3 were designated as *PpSFBB*$^{2\text{-u1}}$, *PpSFBB*$^{2\text{-u2}}$, *PpSFBB*$^{2\text{-u3}}$, *PpSFBB*$^{2\text{-u4}}$, and *PpSFBB*$^{2\text{-u5}}$, and ORF24, ORF31, ORF43, ORF46, and ORF49 were designated as *PpSFBB*$^{2\text{-d1}}$, *PpSFBB*$^{2\text{-d2}}$, *PpSFBB*$^{2\text{-d3}}$, *PpSFBB*$^{2\text{-d4}}$, and *PpSFBB*$^{2\text{-d5}}$, respectively. These *PpSFBB*^{2} genes around *S_2-RNase* shared variable transcriptional orientations (Fig. 1B). Using ATGC Ver. 4 software, the 378 kb *S_2* BAC contig sequence was searched for *SFBB*-like sequences. The analysis revealed two pseudogenes (Ψ*PpSFBB*$^{2\text{-u1}}$ and Ψ*PpSFBB*$^{2\text{-u2}}$) encoding truncated F-box proteins that were located ~46 kb upstream and ~159 kb upstream of *S_2-RNase*, respectively (Fig. 1B). *PpSFBB*$^{2\text{-u1–u5}}$ and *PpSFBB*$^{2\text{-d1–d5}}$ shared 66.3–86.0% amino acid sequence identity with each other, and showed 66.2–93.1% identity with *PpSFBB*$^{4\text{-u1–u4}}$ and *PpSFBB*$^{4\text{-d1–d2}}$ (Table 3).

Total RNA was extracted from pollen, pistils, and leaves of the *S_2* homozygote to examine the expression of *PpSFBB*$^{2\text{-u1–u5}}$ and *SFBB*$^{2\text{-d1–d5}}$. RT-PCR analyses were conducted using gene-specific primer pairs (Supplementary Table S2 at *JXB* online). *PpSFBB*$^{2\text{-u1–u5}}$ and *PpSFBB*$^{2\text{-d1–d5}}$ were all specifically expressed in pollen, but not in pistils or leaves (Supplementary Fig. S1B). Thus, in the 378 kb sequence around *S_2-RNase* there were 10 F-box protein genes (*PpSFBB*$^{2\text{-u1–u5}}$ and *PpSFBB*$^{2\text{-d1–d5}}$) with pollen-specific expression. The *PpSFBB*$^{2\text{-}\gamma}$ gene, previously shown to be linked to the *S_2-RNase*, was not within the sequenced region.

Comparison of deduced amino acid sequences between the PpSFBB^4 and PpSFBB^2 genes

The pairwise deduced amino acid sequence identities of nine *PpSFBB*^{4} genes (*PpSFBB*$^{4\text{-u1–u4}}$, *PpSFBB*$^{4\text{-d1–d2}}$, and *PpSFBB*$^{4\text{-}\alpha\text{–}\gamma}$) and 11 *PpSFBB*^{2} genes (*PpSFBB*$^{2\text{-u1–u5}}$, *PpSFBB*$^{2\text{-d1–d5}}$, and *PpSFBB*$^{2\text{-}\gamma}$) were compared within and between haplotypes (Table 3). Sequence identity among the *PpSFBB*^{4} genes ranged from 62.3% to 86.2%, and among *PpSFBB*^{2} genes ranged from 63.0% to 86.0%. The *PpSFBB*^{4} and *PpSFBB*^{2} genes showed 62.1–99.0% identity between haplotypes. Identities of >90% were found between *PpSFBB*$^{4\text{-u2}}$ and *PpSFBB*$^{2\text{-u1}}$ (92.1%), *PpSFBB*$^{4\text{-u3}}$ and *PpSFBB*$^{2\text{-u2}}$ (93.1%), *PpSFBB*$^{4\text{-}\beta}$ and *PpSFBB*$^{2\text{-u4}}$ (94.9%), and *PpSFBB*$^{4\text{-}\gamma}$ and *PpSFBB*$^{2\text{-}\gamma}$ (99.0%). Identities ranging from 80% to 90% were found between *PpSFBB*$^{4\text{-u2}}$ and *PpSFBB*$^{2\text{-u2}}$ (87.8%), *PpSFBB*$^{4\text{-u3}}$ and *PpSFBB*$^{2\text{-u1}}$ (84.9%), *PpSFBB*$^{4\text{-d1}}$ and *PpSFBB*$^{2\text{-d3}}$ (82.8%), *PpSFBB*$^{4\text{-d2}}$ and *PpSFBB*$^{2\text{-d4}}$ (84.2%), *PpSFBB*$^{2\text{-u1}}$ and *PpSFBB*$^{4\text{-}\alpha}$ (81.9%), and *PpSFBB*$^{2\text{-u2}}$ and *PpSFBB*$^{4\text{-}\alpha}$ (81.6%). The other 89 pairwise comparisons showed identities of <80%.

Phylogenetic analysis of the F-box protein genes of Pyrus and Malus

Most *PpSFBB*^{4} and *PpSFBB*^{2} genes cloned in this study shared the highest amino acid sequence identities with the F-box protein genes of *Malus* (*MdSFBB* and *MdSLF* genes), although *PpSFBB*$^{4\text{-u4}}$ and *PpSFB*$^{2\text{-u4}}$ showed the highest identities with *PpSFBB*$^{3,\ 4,\ 9\text{-}\gamma}$ (77.5%) and *PpSFBB*$^{4\text{-}\beta}$ (94.9%) derived from the same species, respectively

Fig. 3. Alignment of deduced amino acid sequences of *PpSFBB*$^{4\text{-u1–u4},\ 4\text{-d1–d2}}$ and *PpSFBB*$^{2\text{-u1–u5},\ 2\text{-d1–d5}}$. Amino acid sequences were aligned using ClustalW. Conserved sites and relatively conserved sites are marked with asterisks and dots, respectively. F-box domains and FBA_1 domains of F-box proteins are coloured and underlined, respectively. Accession numbers for the F-box protein genes are as follows: *PpSFBB*$^{4\text{-u1–u4},\ 4\text{-d1–d2}}$ (AB545981) and *PpSFBB*$^{2\text{-u1–u5},\ 2\text{-d1–d5}}$ (AB545982).

Table 3. *Pairwise amino acid sequence identities (%) of* PpSFBB^{4} *and* PpSFBB^{2} *genes*

	PpSFBB$^{4\text{-u2}}$	**PpSFBB$^{4\text{-u3}}$**	**PpSFBB$^{4\text{-u4}}$**	**PpSFBB$^{4\text{-d1}}$**	**PpSFBB$^{4\text{-d2}}$**	**PpSFBB$^{4\text{-}\alpha}$**	**PpSFBB$^{4\text{-}\beta}$**	**PpSFBB$^{4\text{-}\gamma}$**	**PpSFBB$^{2\text{-u1}}$**	**PpSFBB$^{2\text{-u2}}$**	**PpSFBB$^{2\text{-u3}}$**	**PpSFBB$^{2\text{-u4}}$**	**PpSFBB$^{2\text{-u5}}$**	**PpSFBB$^{2\text{-d1}}$**	**PpSFBB$^{2\text{-d2}}$**	**PpSFBB$^{2\text{-d3}}$**	**PpSFBB$^{2\text{-d4}}$**	**PpSFBB$^{2\text{-d5}}$**	**PpSFBB$^{2\text{-}\gamma}$**
PpSFBB$^{4\text{-u1}}$	71.4	70.6	72.7	73.1	71.8	69.6	71.0	66.3	72.3	73.6	73.3	71.2	74.0	72.8	76.3	72.6	76.3	71.5	66.3
PpSFBB$^{4\text{-u2}}$	–	86.2	73.7	67.3	69.7	83.2	67.0	70.8	**92.1**	87.8	71.5	66.5	68.5	70.3	72.4	72.0	75.1	66.2	70.3
PpSFBB$^{4\text{-u3}}$		–	70.3	67.2	69.5	80.5	67.2	67.4	84.9	**93.1**	70.1	66.9	68.8	70.8	70.6	72.4	73.7	67.1	66.9
PpSFBB$^{4\text{-u4}}$			–	69.7	68.8	70.9	67.9	77.5	71.2	73.2	68.4	67.1	70.4	70.2	70.4	69.7	72.7	69.4	77.3
PpSFBB$^{4\text{-d1}}$				–	71.2	70.7	69.6	63.6	68.7	68.7	68.8	68.6	72.5	73.9	72.3	82.8	74.3	73.1	63.6
PpSFBB$^{4\text{-d2}}$					–	69.2	67.9	63.7	67.6	70.7	69.7	68.1	71.2	77.4	73.0	74.8	84.2	73.6	63.4
PpSFBB$^{4\text{-}\alpha}$						–	68.7	68.2	81.9	81.6	70.2	68.7	70.7	69.4	70.5	72.5	73.0	69.2	67.7
PpSFBB$^{4\text{-}\beta}$							–	62.3	66.6	68.4	68.8	**94.9**	69.2	71.8	69.0	71.3	73.0	70.5	62.1
PpSFBB$^{4\text{-}\gamma}$								–	68.2	70.0	64.9	62.5	65.5	65.6	65.1	65.1	69.2	65.5	**99.0**
PpSFBB$^{2\text{-u1}}$									–	86.0	69.7	66.3	69.7	70.5	72.0	70.5	73.3	67.9	67.7
PpSFBB$^{2\text{-u2}}$										–	70.5	67.9	70.7	72.8	72.8	74.1	75.1	70.2	69.5
PpSFBB$^{2\text{-u3}}$											–	69.5	70.7	74.1	71.1	72.3	73.8	71.0	64.9
PpSFBB$^{2\text{-u4}}$												–	68.4	70.8	69.0	70.9	72.5	70.0	63.0
PpSFBB$^{2\text{-u5}}$													–	71.0	74.0	72.8	74.3	71.8	65.5
PpSFBB$^{2\text{-d1}}$														–	72.8	75.4	81.2	79.0	65.4
PpSFBB$^{2\text{-d2}}$															–	74.9	77.8	73.6	64.3
PpSFBB$^{2\text{-d3}}$																–	77.1	73.6	65.1
PpSFBB$^{2\text{-d4}}$																	–	78.7	69.4
PpSFBB$^{2\text{-d5}}$																		–	65.3

Values >90% are shown in bold.

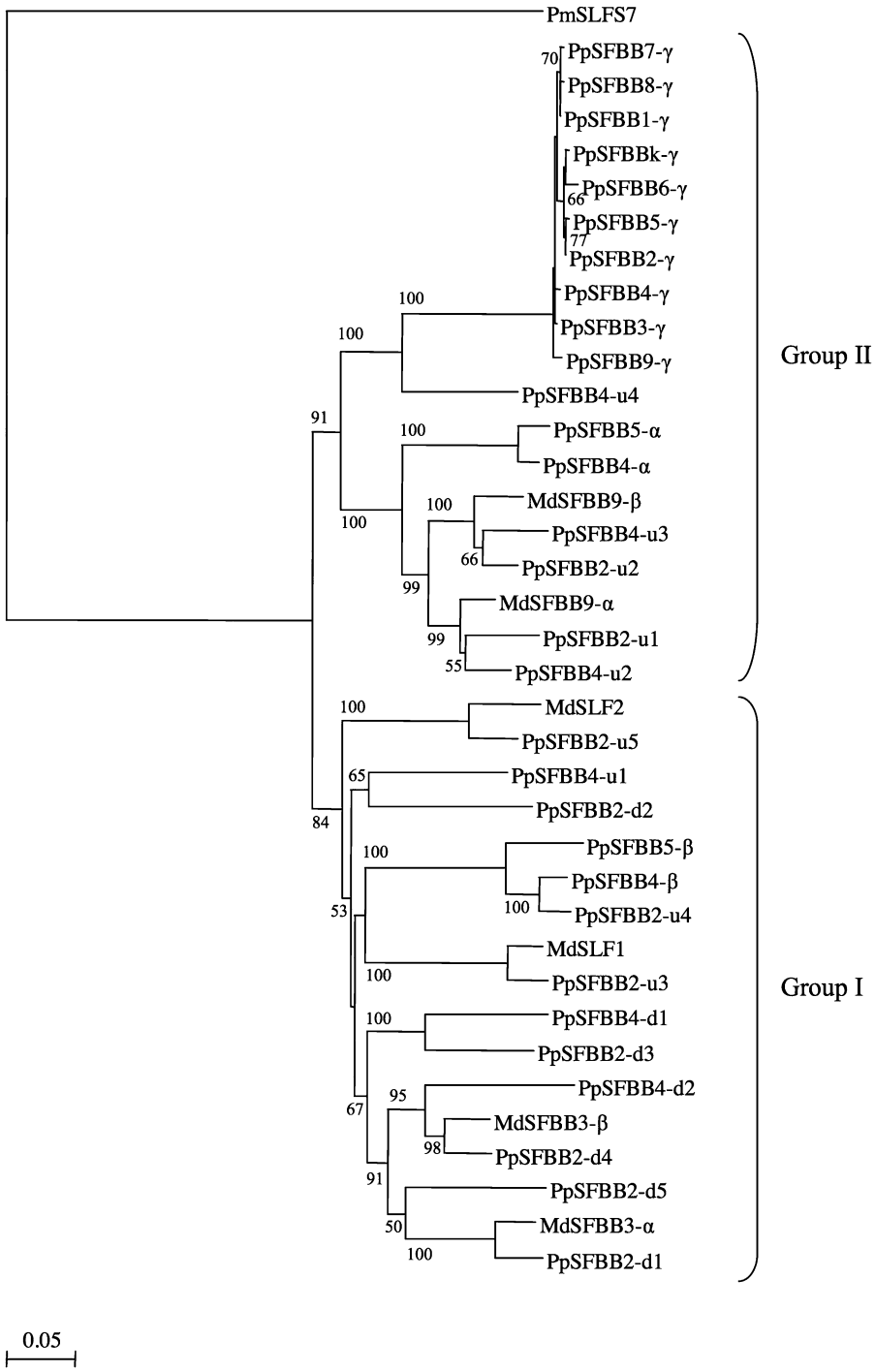


Fig. 4. Phylogenetic analysis of the F-box protein genes of *Pyrus* and *Malus*, and Japanese apricot *PmSLFS*[7]. The phylogenetic tree was constructed using the Neighbor–Joining method. *PmSLFS*[7] was used as an outgroup. Numbers besides the branches are bootstrap values >50%. The bar under the tree represents the number of amino acid substitutions per site.

(Supplementary Table S3 at *JXB* online). The deduced amino acid sequences of the 36 F-box protein genes of *Pyrus* and *Malus* were aligned with *PmSLFS*[7] of *P. mume* using ClustalW, and a rooted phylogenetic tree was constructed by the Neighbor–Joining method with *PmSLFS*[7] as an outgroup (Fig. 4). F-box protein genes of *Pyrus* and *Malus* did not form taxa-independent clusters, and several *PpSFBB* genes were positioned closest to *MdSFBB* and *MdSLF* genes. The F-box protein genes of *Pyrus* and *Malus* were grouped into two major groups: group I (84% bootstrap value) and group II (91% bootstrap value). Group I included *PpSFBB*$^{4\text{-u1, 4-d1–d2}}$, *PpSFBB*$^{2\text{-u3–u5, 2-d1–d5}}$, *PpSFBB*$^{-\beta}$ genes, *MdSFBB*[3] genes, and *MdSLF* genes, while group II included *PpSFBB*$^{4\text{-u2–u4}}$, *PpSFBB*$^{2\text{-u1–u2}}$, *PpSFBB*$^{-\alpha}$ genes, *PpSFBB*$^{-\gamma}$ genes, and *MdSFBB*[9] genes. Comparing group I with group II, amino acid sequences were conserved in F-box domains, but were divergent in the five regions designated as R1, R2, R3, R4, and R5. In these regions, sequences and/or

insertions/deletions (indels) were relatively conserved within each group (Fig. 3).

Discussion

The results of a previous study suggested that the pollen S_4 allele is distal to the region from 48 kb upstream to 188 kb downstream of *S_4-RNase* (Okada *et al.*, 2008). In this study, the BAC contig around *S_4-RNase* was extended to 659 kb, and a 648 516 bp region spanning 290 kb upstream to 359 kb downstream of *S_4-RNase* was sequenced. Sequence analysis of the 649 kb region predicted five new pollen-specific F-box protein genes (*PpSFBB*$^{4\text{-u1–u4}}$ and *PpSFBB*$^{4\text{-d2}}$). The 649 kb sequence around *S_4-RNase* included six *PpSFBB*^{4} genes including *PpSFBB*$^{4\text{-d1}}$ (*S_4F-box0*), but not *PpSFBB*$^{4\text{-}\alpha\text{-}\gamma}$. In addition, a BAC library was constructed from 'Choujuuro' (S_2S_3), and a BAC contig of 391 kb around *S_2-RNase* was assembled. Sequence analysis of a 378 419 bp region spanning 166 kb upstream to 212 kb downstream of *S_2-RNase* predicted 10 new pollen-specific F-box protein genes (*PpSFBB*$^{2\text{-u1–u5, 2-d1–d5}}$). The 378 kb sequence around *S_2-RNase* included 10 *PpSFBB*^{2} genes, but not *PpSFBB*$^{2\text{-}\gamma}$. The predicted products of *PpSFBB*$^{4\text{-u1–u4, 4-d1–d2}}$, and *PpSFBB*$^{2\text{-u1–u5, 2-d1–d5}}$ showed typical features of F-box proteins: an F-box domain at the N-terminus and an FBA_1 domain in the centre (Fig. 3). These results indicated that F-box protein genes with pollen-specific expression are clustered around the *S-RNase* of Japanese pear, and that *PpSFBB*$^{4\text{-}\alpha\text{-}\gamma}$ and *PpSFBB*$^{2\text{-}\gamma}$, which are linked to the *S-RNase*, were located outside the sequenced region.

Organization of the F-box protein gene cluster around the S-RNase *gene of Japanese pear*

Among *PpSFBB*$^{4\text{-u1–u4, 4-d1–d2}}$ and *PpSFBB*$^{2\text{-u1–u5, 2-d1–d5}}$, some genes may be located more distantly from *S*-locus regions. Entani *et al.* (2003) conducted pattern matching analysis of homologies (Harr plot analysis) for the sequences around *PmS_1-* and *PmS_7-RNases* of *P. mume.* Their results revealed that highly divergent *S_1-* and *S_7*-locus regions are surrounded by co-linear flanking regions, and that *S_1-* and *S_7*-locus regions are ~27 kb and 15 kb long, respectively. Harr plot analysis of the 649 kb and 378 kb sequences around *S_4-* and *S_2-RNases* was conducted, and no co-linearity was found between these sequences (data not shown). This result suggests that both the 649 kb and 378 kb sequences are a part of the *S*-locus region, or that either sequence could contain both the *S*-locus region and its flanking region. Sequence analysis of the 649 kb and 378 kb regions predicted 40 and 20 transposon-like sequences around *S_4-RNase* and *S_2-RNase*, respectively (Tables 1, 2). The *S*-locus, which controls S-RNase-based GSI, contains many transposon-like sequences. For example, transposon-like sequences were found in three out of 12 ORFs in 72 kb of the *P. dulcis* S_c-haplotype (Ushijima *et al.*, 2003), in four out of 11 ORFs in 64 kb of the *A. hispanicum* *S_2*-haplotype (Lai *et al.*, 2002), and in 31 out of 50 ORFs in 328 kb of the *P. inflata* *S_2*-haplotype (Wang *et al.*, 2004). These transposon-like sequences generate polymorphisms among *S*-haplotypes, and might contribute to suppression of recombination between *S-RNase* and *SLF/SFB*. In the sequenced regions around *S_4-RNase* and *S_2-RNase*, the non-co-linearity, the abundant (retro) transposon insertions, and the absence of *PpSFBB*$^{4\text{-}\alpha\text{-}\gamma}$ and *PpSFBB*$^{2\text{-}\gamma}$ suggest that the 649 kb and 378 kb sequences around *S_4-RNase* and *S_2-RNase* are part of the *S*-locus region, and that the *S*-locus regions of the Japanese pear are probably larger than those of *Prunus* species.

The organization of the F-box protein gene clusters around the *S_4-RNase* and *S_2-RNase* was compared when *S_4-RNase* and *S_2-RNase* were fixed in the same transcriptional orientation (Fig. 1). *PpSFBB*$^{4\text{-u1}}$ and *PpSFBB*$^{4\text{-d1}}$ are located ~113 kb upstream and ~127 kb downstream of *S_4-RNase*, whereas *PpSFBB*$^{2\text{-u1}}$ and *PpSFBB*$^{2\text{-d1}}$ are located close to *S_2-RNase* (~22 kb upstream and ~10 kb downstream of *S_2-RNase*, respectively). The average densities of F-box protein genes were one gene/108 kb around *S_4-RNase* and one gene/38 kb around *S_2-RNase*. Together, these results suggest that F-box protein genes are clustered in the region around *S_2-RNase* more tightly than in the region around *S_4-RNase.*

F-box protein genes, *SLF/SFB* and SLF-like genes (*SLFL*), were identified in cosmid and fosmid contigs around the *S-RNase* of *Prunus* species. *SLF/SFB* genes are the pollen *S* genes, but *SLFL* genes are probably not involved in SI recognition (Entani *et al.*, 2003; Ushijima *et al.*, 2003). *SLF/SFB* and *SLFL1–SLFL3* cloned from the same haplotypes show low amino acid sequence identity with each other. For example, *PmSLFS*^{7} is 11.7–16.9% identical to *PmSLFL1S*^{7}, *PmSLFL2S*^{7}, and *PmSLFL3S*^{7}, which share 26.9–45.3% identity with each other (Entani *et al.*, 2003; Matsumoto *et al.*, 2008). *PdSFB*^{c} and *PdSFB*^{d} are 18.7 and 20.2% identical to *PdSLF*^{c} and *PdSLF*^{d} (orthologuess of *PmSLFL1* of *P. mume*), respectively (Ushijima *et al.*, 2003). In contrast to *Prunus* species, *PpSFBB*$^{4\text{-u1–u4, 4–d1–d2}}$ and *PpSFBB*$^{2\text{-u1–u5, 2-d1–d5}}$ shared 67.2–86.2% and 66.3–86.0% identity within each haplotype, respectively (Table 3). This indicates that the region around an *S-RNase* of the Japanese pear comprises related F-box protein genes, which is different from the F-box protein gene organization around the *S-RNases* in *Prunus*, in which there are clusters of F-box protein genes that show low levels of identity to each other. The amino acid sequence identities between *PpSFBB*$^{4\text{-u1–u4, 4-d1–d2}}$ and *PpSFBB*$^{2\text{-u1–u5, 2-d1–d5}}$ ranged from 66.2% to 93.1% (Table 3), and were higher than those within each haplotype (66.3–86.2%). These similarities between haplotypes indicated that related polymorphic F-box protein genes between haplotypes were clustered in the regions around *S_4-RNase* and *S_2-RNase.*

Classification of PpSFBB *genes based on phylogenetic analysis and sequence polymorphism*

In *Prunus* species, F-box protein genes around *S-RNase* genes were grouped into two major classes, the *SLF/SFB* clade and the *SLFL* clade, by a phylogenetic analysis

(Matsumoto *et al.*, 2008). *SLF*/*SFB* genes show lower levels of allelic sequence identity (77.8–81.3% for *PmSLF* genes, 68.4–76.4% for *PdSFB* genes, and 75.1–81.1% for *PavSFB* genes, respectively) than *SLFL* genes (88.5–92.0% for *PmSLFL1*, 95.8–98.6% for *PmSLFL2*, and 95.1% for *PdSLF*) (Entani *et al.*, 2003; Ushijima *et al.*, 2003; Ikeda *et al.*, 2004; Matsumoto *et al.*, 2008). The sequence differences of the F-box protein genes among haplotypes implied that *SLF*/*SFB* genes with lower levels of identity were pollen *S* candidates, and that *SLFL* genes with high levels of identity were not (Entani *et al.*, 2003; Ushijima *et al.*, 2003).

The phylogenetic relationships and sequence differences of F-box protein genes of *Pyrus* and *Malus* would be useful for delineating pollen *S* candidates from $PpSFBB^{4\text{-u1–u4, 4-d1–d2}}$ and $PpSFBB^{2\text{-u1–u5, 2-d1–d5}}$. Phylogenetic analysis based on the deduced amino acid sequences of 36 F-box protein genes of *Pyrus* and *Malus* allowed them to be classified into two major groups, I and II (Fig. 4). $PpSFBB^{4\text{-u2–u4}}$, $PpSFBB^{2\text{-u1–u2}}$, $PpSFBB^{-\alpha}$ genes, $PpSFBB^{-\gamma}$ genes and $MdSFBB^{9}$ genes were classified into group II, and the other *PpSFBB*, $MdSFBB^{3}$, and *MdSLF* genes were in group I. The phylogenetic analysis also generated a $PpSFBB^{-\gamma}$ subgroup and 10 gene pairs of *PpSFBB* genes and *PpSFBB*/*Malus* F-box protein genes. Sequence identities between the paired genes ranged from 76.3% to 96.4% (Table 3, Supplementary Table S3 at *JXB* online), which were higher than those among *PpS_2*-, *PpS_4*-, *PpS_5*-, *MdS_1*-, *MdS_2*-, *MdS_3*-, and *MdS_9-RNases* (60.9–71.1%). Group I consisted of gene pairs with high levels of identity (>91%): $MdSLF2/PpSFBB^{2\text{-u5}}$ (91.3% identity), $PpSFBB^{4\text{-}\beta}/PpSFBB^{2\text{-u4}}$ (94.9% identity), $MdSLF1/PpSFBB^{2\text{-u3}}$ (93.9% identity), $MdSFBB^{3\text{-}\beta}/PpSFBB^{2\text{-d4}}$ (93.1% identity), $MdSFBB^{3\text{-}\alpha}/PpSFBB^{2\text{-d1}}$ (92.9% identity); and gene pairs with low levels of identity: $PpSFBB^{4\text{-u1}}/PpSFBB^{2\text{-d2}}$ (76.3% identity) and $PpSFBB^{4\text{-d1}}/PpSFBB^{2\text{-d3}}$ (82.8% identity). Group II consisted of the $PpSFBB^{-\gamma}$ subgroup sharing 97.5–99.7% identity among 10 haplotypes (Kakui *et al.*, 2007), and gene pairs with high levels of identities (>92%): $PpSFBB^{5\text{-}\alpha}/PpSFBB^{4\text{-}\alpha}$ (96.4% identity, Sassa *et al.*, 2007), $PpSFBB^{4\text{-u3}}/PpSFBB^{2\text{-u2}}$ (93.1% identity), and $PpSFBB^{2\text{-u1}}/PpSFBB^{4\text{-u2}}$ (92.1% identity). The gene pairs with low levels of identity were included in group I, not in group II, suggesting that pollen *S* candidates were included in the group I.

Therefore, the group I F-box protein genes from the region around *S-RNase* with low levels of sequence identity, $PpSFBB^{4\text{-u1}}/PpSFBB^{2\text{-d2}}$ and $PpSFBB^{4\text{-d1}}/PpSFBB^{2\text{-d3}}$, are expected to be pollen *S* candidates of Japanese pear. In a previous study, $PpSFBB^{4\text{-d1}}$ (*S_4F-box0*) was thought unlikely to be the pollen *S_4* allele, because it is found in the deleted region of the S_4^{sm}-haplotype (Okada *et al.*, 2008). Interestingly, Saito *et al.* (2002) observed that S_4^{sm} pollen is rejected by pistils harbouring not only the *S_4*-haplotype, but also the *S_1*-haplotype. It seems, therefore, that S_4^{sm} pollen has a dual specificity for S_4-RNase and S_1-RNase, which have high amino acid identity (90.0%) (Ishimizu *et al.*, 1998). This dual specificity is probably due to the loss of $PpSFBB^{4\text{-d1}}$, and S_4^{sm} pollen might come to recognize S_1-RNase. Therefore, $PpSFBB^{4\text{-d1}}$ might also be a pollen *S* candidate. However, *SLF* genes of *A. hispanicum* and *P. inflata* share high levels of amino acid identity among haplotypes (97–99% and 88.4–89.7%, respectively; Zhou *et al.*, 2003; Sijacic *et al.*, 2004). There is no evidence for a co-evolutionary relationship between *SLF*/*SFB* and *S-RNase* in *A. hispanicum* and *P. inflata*, or in *Prunus* species, which implies that sequence polymorphism between haplotypes can no longer be considered a reliable diagnostic feature of pollen *S* genes, and functional analysis must be used to identify pollen *S* genes (Newbigin *et al.*, 2008). Therefore, all $PpSFBB^{4}$ genes and $PpSFBB^{2}$ genes should be considered as pollen *S* gene candidates.

However, it is not a reasonable interpretation that all *PpSFBB* genes act in concert as the pollen *S* genes. Among several F-box protein genes around the *S-RNases* of *Prunus* species, *A. hispanicum*, and *P. inflata*, one F-box protein gene, *SLF*/*SFB*, functions as the pollen *S* gene; the other F-box protein genes, *SLFL* genes, are non-*S* pollen genes (Entani *et al.*, 2003; Ushijima *et al.*, 2003; Zhou *et al.*, 2003; Wang *et al.*, 2004; Hua *et al.*, 2007). Therefore, several *PpSFBB* genes in a particular haplotype are probably not pollen *S* genes. The non-pollen *S* proteins of *P. inflata*, PiSLFLs, either fail to interact with S-RNase or interact much more weakly than PiSLF. When the deduced amino acid sequences of PiSLF and PiSLFLs were compared, three PiSLF-specific regions (SR1, SR2, and SR3) that confer on PiSLF its unique function in SI were revealed (Hua *et al.*, 2007). Although the interactions of PpSFBBs with S-RNase have not yet been analysed, five regions (R1, R2, R3, R4, and R5) were identified where amino acid sequences were variable between the group I and II F-box proteins (Fig. 3). The sequence differences in these regions might account for different interactions with S-RNase between the group I and II F-box proteins. Therefore, there remains the possibility that the less polymorphic group II F-box protein genes are non-pollen *S* genes.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Expression of *PpSFBB* genes located around *S_4-RNase* (A) and *S_2-RNase* (B).

Table S1. Primer pairs used to construct BAC contigs around *S-RNase.*

Table S2. Gene-specific RT-PCR primer pairs.

Table S3. Pairwise amino acid sequence identities (%) of *PpSFBB* genes with previously reported *PpSFBB*, *MdSFBB*, and *MdSLF* genes.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research (B) (nos 16380028; 19380020) and (C) (no. 17580027) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. K.O. was supported by research fellowships of the Japan Society for the Promotion of Science for Young Scientists.

References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389–3402.

Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* **9,** 208–218.

Burge C, Karlin S. 1997. Prediction of complete gene structures in human genomic DNA. *Journal of Molecular Biology* **268,** 78–94.

Castillo C, Takasaki T, Saito T, Yoshimura Y, Norioka S, Nakanishi T. 2001. Reconsideration of *S*-genotype assignments, and discovery of a new allele based on *S-RNase* PCR-RFLPs in Japanese pear cultivars. *Breeding Science* **51,** 5–11.

Cheng JH, Han ZH, Xu XF, Li TZ. 2006. Isolation and identification of the pollen-expressed polymorphic F-box genes linked to the *S*-locus in apple (*Malus*×*domestica*). *Sexual Plant Reproduction* **19,** 175–183.

de Nettancourt D. 2001. *Incompatibility and incongruity in wild and cultivated plants*. Berlin: Springer-Verlag.

Entani T, Iwano M, Shiba H, Che FS, Isogai A, Takayama S. 2003. Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: identification of a pollen-expressed F-box gene with allelic diversity. *Genes to Cells* **8,** 203–213.

Hauck NR, Ikeda K, Tao R, Iezzoni AF. 2006. The mutated S_1-haplotype in sour cherry has an altered *S*-haplotype-specific F-box protein gene. *Journal of Heredity* **97,** 514–520.

Hua ZH, Meng XY, Kao T- H. 2007. Comparison of *Petunia inflata* *S*-locus F-box protein (Pi SLF) with Pi SLF-like proteins reveals its unique function in S-RNase based self-incompatibility. *The Plant Cell* **19,** 3593–3609.

Huang S, Lee HS, Karunanandaa B, Kao TH. 1994. Ribonuclease activity of *Petunia inflata* *S*-proteins is essential for rejection of self-pollen. *The Plant Cell* **6,** 1021–1028.

Ikeda K, Igic B, Ushijima K, Yamane H, Hauck NR, Nakano R, Sassa H, Iezzoni AF, Kohn JR, Tao R. 2004. Primary structural features of the *S*-haplotype-specific F-box protein, SFB *in Prunus*. *Sexual Plant Reproduction* **16,** 235–243.

Ishimizu T, Inoue K, Shimonaka M, Saito T, Terai O, Norioka S. 1999. PCR-based method for identifying the *S*-genotypes of Japanese pear cultivars. *Theoretical and Applied Genetics* **98,** 961–967.

Ishimizu T, Sato Y, Saito T, Yoshimura Y, Norioka S, Nakanishi T, Sakiyama F. 1996. Identification and partial amino acid sequences of seven S-RNases associated with self-incompatibility of Japanese pear, *Pyrus pyrifolia* Nakai. *Journal of Biochemistry* **120,** 326–334.

Ishimizu T, Shinkawa T, Sakiyama F, Norioka S. 1998. Primary structural features of rosaceous S-RNases associated with gametophytic self-incompatibility. *Plant Molecular Biology* **37,** 931–941.

Kakui H, Tsuzuki T, Koba T, Sassa H. 2007. Polymorphism of SFBB-gamma and its use for *S* genotyping in Japanese pear (Pyrus pyrifolia). *Plant Cell Reports* **26,** 1619–1625.

Lai Z, Ma WS, Han B, Liang LZ, Zhang YS, Hong GF, Xue YB. 2002. An F-box gene linked to the self-incompatibility (*S*) locus of Antirrhinum is expressed specifically in pollen and tapetum. *Plant Molecular Biology* **50,** 29–42.

Lechner E, Achard P, Vansiri A, Potuschak T, Genschik P. 2006. F-box proteins everywhere. *Current Opinion in Plant Biology* **9,** 631–638.

Matsumoto D, Yamane H, Tao R. 2008. Characterization of *SLFL1*, a pollen-expressed F-box gene located in the *Prunus S* locus. *Sexual Plant Reproduction* **21,** 113–121.

McClure BA, Franklin-Tong V. 2006. Gametophytic self-incompatibility: understanding the cellular mechanisms involved in 'self' pollen tube inhibition. *Planta* **224,** 233–245.

McClure BA, Gray JE, Anderson MA, Clarke AE. 1990. Self-incompatibility in *Nicotiana alata* involves degradation of pollen ribosomal RNA. *Nature* **347,** 757–760.

McClure BA, Haring V, Ebert PR, Anderson MA, Simpson RJ, Sakiyama F, Clarke AE. 1989. Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* **342,** 955–957.

McCubbin AG, Kao TH. 2000. Molecular recognition and response in pollen and pistil interactions. *Annual Review of Cell and Developmental Biology* **16,** 333–364.

Newbigin E, Paape T, Kohn JR. 2008. RNase-based self-incompatibility: puzzled by pollen S. *The Plant Cell* **20,** 2286–2292.

Okada K, Tonaka N, Moriya Y, Norioka N, Sawamura Y, Matsumoto T, Nakanishi T, Takasaki-Yasuda T. 2008. Deletion of a 236 kb region around S_4*-RNase* in a stylar-part mutant $S4^{sm}$-haplotype of Japanese pear. *Plant Molecular Biology* **66,** 389–400.

Potter D, Eriksson T, Evans RC, *et al*. 2007. Phylogeny and classification of Rosaceae. *Plant Systematics and Evolution* **266,** 5–43.

Saito T, Sato Y, Sawamura Y, Shoda M, Kotobuki K. 2002. Studies on breeding of self-compatibility in Japanese pear. 2. Characteristic of pollen of $S4^{sm}$ gene originated from 'Osa-nijisseiki'. Journal of the Japanese Society for Horticultural Science 71. *suppl* **2),** 123(in Japanese).

Saitou N, Nei M. 1987. The neighbor–joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4,** 406–425.

Sassa H, Kakui H, Miyamoto M, Suzuki Y, Hanada T, Ushijima K, Kusaba M, Hirano H, 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,** 1869–1881.

Sijacic P, Wang X, Skirpan AL, Wang Y, Dowd PE, McCubbin AG, Huang S, Kao TH. 2004. Identification of the pollen determinant of S-RNase-mediated self-incompatibility. *Nature* **429,** 302–305.

Sato Y. 1993. Breeding of self-compatible Japanese pear. In: Hayashi T, Omura M, Scott NS, eds. *Techniques on gene diagnosis and breeding in fruit trees*. 241–247 Tsukuba, Japan: FTRS (Fruit Tree Research Station).

Sonneveld T, Tobutt KR, Vaughan SP, Robbins TP. 2005. Loss of pollen *S* function in two self-compatible selections of *Prunus avium* is associated with deletion/mutation of an *S* haplotype-specific F-box gene. *The Plant Cell* **17,** 37–51.

Terai O, Sato Y, Saito T, Abe K, Kotobuki K. 1999. Identification of homozygotes of self-incompatibility gene (*S*-gene), as useful tools to determine the *S*-genotype in Japanese pear, *Pyrus pyrifolia* Nakai. *Bulletin of the National Institute of Fruit Tree Science* **32,** 31–38.

Tsukamoto T, Hauck NR, Tao R, Jiang N, Iezzoni AF. 2006. Molecular characterization of three non-functional *S*-haplotypes in sour cherry (*Prunus cerasus*). *Plant Molecular Biology* **62,** 371–383.

Ushijima K, Sassa H, Dandekar AM, Gradziel TM, Tao R, Hirano H. 2003. Structural and transcriptional analysis of the self-incompatibility locus of almond: identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. *The Plant Cell* **15,** 771–781.

Ushijima K, Yamane H, Watari A, Kakehi E, Ikeda K, Hauck NR, Iezzoni AF, Tao RT. 2004. The *S* haplotype-specific F-box protein gene, *SFB*, is defective in self-compatible haplotypes of *Prunus avium* and *P. mume*. *The Plant Journal* **39,** 573–586.

Vilanova S, Badenes ML, Burgos L, Martinez-Calvo J, Llacer G, Romero C. 2006. Self-compatibility of two apricot selections is associated with two pollen-part mutations of different nature. *Plant Physiology* **142,** 629–641.

Wang Y, Tsukamoto T, Yi KW, Wang X, Huang SS, McCubbin AG, Kao TH. 2004. Chromosome walking in the *Petunia inflata* self-incompatibility (*S*) locus and gene identification in an 881 kb contig containing S_2*-RNase*. *Plant Molecular Biology* **54,** 727–742.

Xue YB, Carpenter R, Dickinson HG, Coen ES. 1996. Origin of allelic diversity in antirrhinum *S* locus RNases. *The Plant Cell* **8,** 805–814.

Zhou JL, Wang F, Ma WS, Zhang YS, Han B, Xue YB. 2003. Structural and transcriptional analysis of *S*-locus F-box genes in. *Antirrhinum. Sexual Plant Reproduction* **16,** 165–177.