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ORIGINAL PAPER

Cloning and mapping multiple S-locus F-box genes in European pear (*Pyrus communis* L.)

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Abstract European pear, as well as its close relatives Japanese pear and apple, exhibits S-RNase-based gametophytic self-incompatibility. The male determinant of this self-incompatibility mechanism is a pollen-expressed protein containing an F-box domain; in the genera *Petunia* (Solanaceae), *Antirrhinum* (Plantaginaceae), and *Prunus* (Rosaceae), a single F-box gene determines the pollen S. In apple and Japanese pear, however, multiple S-locus F-box genes were recently identified as candidates for the pollen S, and they were named S-locus F-Box Brothers. These genes were considered good candidates for the pollen S determinant since they exhibit S-haplotype-specific polymorphisms, pollen-specific expression, and linkage to the *S-RNase*. In the present study, S-locus F-Box Brothers homologs have been cloned from two of the most agronomically important European pear varieties, "Abbé Fétel" ($S_{104\text{-}2}/S_{105}$) and "Max Red Bartlett" (S_{101}/S_{102}), and they have been mapped on a genetic linkage map developed on their progeny. Our results suggest that the number of F-box genes linked to the S-locus of the European pear is higher than expected according with previous reports for apple and Japanese pear, since up to five genes were found to be linked to a single S-haplotype. Moreover, two of these genes exhibited an incomplete linkage to the *S-RNase*, allowing the identification of low-frequency recombinant haplotypes, generated by a crossing-over event between the two genes. These F-box genes are most likely placed in close proximity of the S-locus but do not belong to it, and they can thus be excluded from being responsible for the determination of pollen S function.

Keywords Pear · S-locus · *S-RNase* · *SFBB* · *SFB*

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Introduction

European pear (*Pyrus communis* L.) belongs to the family Rosaceae, which includes many of the most important cultivated fruit species. The great majority of the Rosaceae are hermaphrodite, and they carry perfect flowers; however, in many genera, self-fertilization is prevented by a gametophytic self-incompatibility (GSI) system, which acts through a specific pollen-pistil recognition mechanism. The pollen tubes that are recognized as "self" are blocked at the upper part of the style, whereas the "non-self" (compatible) pollen tubes are allowed to grow along the style and reach the ovary. The specificity of this recognition mechanism is genetically controlled by a single, multiallelic locus (S-locus). The S-locus harbors at least two genes, coding for a female and a male determinant that are specifically expressed in the pistil and pollen respectively. A pollen tube is rejected whenever its haploid genome carries a pollen specificity that matches one of the two specificities expressed by the pistil tissue (De Nettancourt 2001).

In this ancestral and widespread self-incompatibility system, described not only in the Rosaceae but also in the distantly related families Solanaceae and Plantaginaceae

(De Nettancourt 2001), the female determinant is a stylar glycoprotein with ribonuclease activity, the S-RNase. Since its discovery in the 1980s (Bredemeijer and Blaas 1981; Anderson et al. 1986; McClure et al. 1989), this protein and its coding gene have been intensively studied in different species. In the European pear, the *S-RNase* gene has been sequenced for 24 different S-haplotypes, and several S-genotyping molecular assays have been developed on this gene (Zuccherelli et al. 2002; Zisovich et al. 2004; Sanzol et al. 2006; Takasaki et al. 2006; Moriya et al. 2007; Mota et al. 2007; Sanzol and Robbins 2008; Sanzol 2009a, b; Goldway et al. 2009; Sanzol 2010).

On the other hand, the male determinant of S-RNase-based GSI has been only recently identified as a pollen protein containing an F-box domain in its amino-terminal portion, named *S-L*ocus *F*-box (SLF; Lai et al. 2002) or *S*-haplotype-specific *F-B*ox (SFB; Ushijima et al. 2003). The *SLF* gene was first identified after genomic analyses of the S-locus region in *Antirrhinum* (Lai et al. 2002), and the demonstration of its role as the pollen determinant of S-RNase-based GSI came in 2004 after transformation experiments in *Petunia* (Sijacic et al. 2004) and *Antirrhinum* (Qiao et al. 2004).

European pear (*P. communis* L.), Japanese pear (*Pyrus pyrifolia* Nakai), and apple (*Malus* × *domestica* Borkh.) belong to the subtribe Pyrinae (formerly the subfamily Maloideae; Potter et al. 2007; Campbell et al. 2007), a taxon in which the research for the GSI male determinant gave results of still uncertain interpretation. In all the species carrying S-RNase-based GSI for which the S-locus determinants have been characterized, the pollen S is the product of a single gene; nevertheless, Sassa et al. (2007) reported for the *Pyrinae* species apple and Japanese pear the identification of multiple S-locus F-box genes, which were named *S*-locus *F-B*ox *B*rothers (*SFBB*). Two *SFBB*s were identified in the genomic region surrounding each of the apple *S-RNase*s S_3 and S_9 ($MdSFBB^{\alpha}$ and $MdSFBB^{\beta}$; *Md* stands for *Malus* × *domestica*), and three *SFBB*s were found associated to each of the S-haplotypes S_4 and S_5 of Japanese pear, from pollen-derived cDNA ($PpSFBB^{\alpha}$, $PpSFBB^{\beta}$, and $PpSFBB^{\gamma}$; *Pp* stands for *P. pyrifolia*). The $PpSFBB^{\gamma}$ gene has been further characterized in other S-haplotypes of Japanese pear, and it has been used for the development of a molecular S-genotyping assay (Kakui et al. 2007). Very recently, Zisovich et al. (2009) published the first S-locus F-box gene sequences from European pear; seven *SFB*s, linked to seven different S-haplotypes, were identified, and also in this case, they were used in a molecular S-genotyping assay. However, no information is available regarding the number of F-box genes belonging to each S-haplotype in this species.

In this article, we describe for the first time the identification of multiple S-locus F-box genes in the European pear. The identified F-box genes include homologs not only to the three Japanese pear *SFBB*s but also to the *SFBB*s of apple, suggesting that the number of F-box genes surrounding the *S-RNase* is higher than expected. The segregation of all the F-box genes was tested in a cross progeny of 92 individuals: all of them resulted tightly linked to the *S-RNase* and were placed on a genetic linkage map on LG 17, where the S-locus has been previously mapped both in apple (Maliepaard et al. 1998) and pear (Yamamoto et al. 2002). However, very rare recombination events between the *S-RNase* and some of the F-box genes could be identified, suggesting that these genes are linked, but do not belong, to the S-locus. The pear S-locus structure is discussed according to reported bibliography and experimental evidences.

Materials and methods

Plant material and DNA extraction

Two European pear cultivars, Abbé Fétel and Max Red Bartlett, and a population consisting of 92 F1 individuals resulting from the cross "Abbé Fétel" × "Max Red Bartlett" were used in this study. The trees were housed at the Experimental Station of the "Dipartimento di Colture Arboree" (Bologna, Italy); the S genotypes of the two parental cultivars are $S_{104\text{-}2}/S_{105}$ for "Abbé Fétel" and S_{101}/S_{102} for "Max Red Bartlett."

For each F1 plant and the two parental varieties, 4 g of fresh young leaves was ground in liquid nitrogen, and DNA was extracted following a modified cetyl trimethyl ammonium bromide (CTAB) protocol (Maguire et al. 1994). DNA concentration and purity were assessed using a Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technology, Rockland, DE).

Primer design, polymerase chain reaction, and sequencing

Polymerase chain reaction (PCR) amplification of S-locus F-box genes was performed using the primers listed in Table 1. Primers were designed on the basis of the sequences of *SFBB* genes reported by Sassa et al. (2007) and tested using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCR amplifications were performed in a MJ PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following conditions: 50 ng genomic DNA, 1× PCR reaction buffer (supplied with the enzyme), 0.2 mM $MgCl_2$, 0.1 mM dNTPs, 0.3 μM each primer, and 1 unit *Taq* DNA polymerase (Amplibiotherm, Fisher Molecular Biology), and with the following program: 2 min of denaturation at 94°C; 36 cycles of 30 s at 94°C, 1 min at 60°C, 2 min at 72°C; and a final extension of 10 min at 72°C.

Table 1 Primers used for the amplification of the *SFBB* genes from "Abbé Fétel" and "Max Red Bartlett" (from De Franceschi et al. submitted)

Primer name	5′–3′ Sequence
PpAlpha forward	TCTTGTGGAATGATACTGC
PpAlpha reverse	ATATCATGCATACAAATTAAATGGAAC
PpBeta forward	GTCCCAGGTGCGTAAAAGTG
PpBeta reverse 2	TTAAATAGGAGAAAATGGAAGTTTGC
PpGamma forward	GTGTGAATAATTCATGTGCATGG
PpGamma reverse	GGAACGTTTCCCTCAACTCAA
Md3 forward	AACCGTTCTCAGGCTCACA
Md3 reverse	CACATAAATAAGAMCTTCAAAATCCAC
Md9Beta forward	ATTCCTGTGGACCAATACAGTTG
Md9 reverse	CCACAAAGCAAAATTAGAAGATGC

For cloning and sequencing, PCR was carried out using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzyme, Espoo, Finland), following the manufacturer's protocol. PCR products were purified with phenol, and the 3′-A overhangs were added by incubating the purified amplicons with 0.5 mM dATP, 1× PCR reaction buffer, and 1 unit of *Taq* DNA polymerase. A-tailed amplifications were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI) and *Escherichia coli* DH5α chemically competent cells. The cloned PCR products were amplified by colony PCR and digested with five different restriction enzymes (*Alu*I, *Bsu*RI, *Mse*I/*Tru*1I, *Rsa*I, and *Taq*I); every clone producing a different restriction profile was selected for sequencing. Sequencing reactions were carried out using M13 forward and SP6 universal primers.

All the cloned sequences were submitted to the National Center for Biotechnology Information (NCBI) database, and their accession numbers are reported in Table 2.

Sequence analysis

The deduced protein sequences of all the *SFBB* genes were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html; Larkin et al. 2007), and the corresponding nucleotide sequences were aligned accordingly. MEGA 4 (Tamura et al. 2007) was used to construct the phylogenetic tree (Fig. 1), using the neighbor-joining (NJ) algorithm with 1,000 bootstraps, and to calculate the genetic distances (Table 3); pairwise p-distances, indicating the proportion of divergent nucleotides between two compared sequences, were chosen for this analysis.

Segregation analysis

S-genotypes of all the individuals of the progeny were determined by PCR using primers designed on the *S-RNase* gene, PycomC1F (5′-ATTTTCAATTTACGCAGCAATA TCAGC-3′) and PycomC5R (5′-CTGCAAAGWSHGACC TCAACCAATTC-3′) according with Sanzol (2009a); the amplified products were resolved using agarose gel electrophoresis.

Specific Cleavage Amplified Polymorphic Sequence (CAPS) markers were developed on the obtained F-box genes sequences to analyze the segregation. The restriction enzymes used for each sequence group and the restriction profiles for the mapped sequences are reported in Table 4; digested amplicons were separated on polyacrylamide or agarose gel electrophoresis.

Mapping

The segregation data for the *S-RNase* and *SFBB* genes were integrated in the map dataset already available for the population "Abbé Fétel" × "Max Red Bartlett" (Pierantoni et al. 2004, 2007; Dondini et al. 2008) and mapped using

Table 2 Summary of the *SFBB* sequences cloned from the cultivars "Abbé Fétel" and "Max Red Bartlett"

Gene	Primer pair	Cloned sequences (accession number)	
		"Abbé Fétel"	"Max Red Bartlett"
SFBBα	PpAlpha for/rev	*SFBBα-AF1* (HM013871)	*SFBBα-MRB1* (HM013877)
			SFBBα-MRB2 (HM013878)
SFBBβ	PpBeta for/rev2	*SFBBβ-AF1* (HM013872)	*SFBBβ-MRB1* (HM013879)
		SFBBβ-AF2 (HM013873)	*SFBBβ-MRB2* (HM013880)
SFBBγ	PpGamma for/rev	*SFBBγ-AF1* (HM013874)	*SFBBγ-MRB1* (HM013881)
		SFBBγ-AF2 (HM013875)	*SFBBγ-MRB2* (HM013882)
SFBBδ	Md3 for/rev	*SFBBδ-AF1* (HM013876)	*SFBBδ-MRB1* (HM013883)
			SFBBδ-MRB2 (HM013884)
SFBBε	Md9Beta for/Md9 rev	No amplification	*SFBBε-MRB1* (HM013885)
			SFBBε-MRB2 (HM013886)

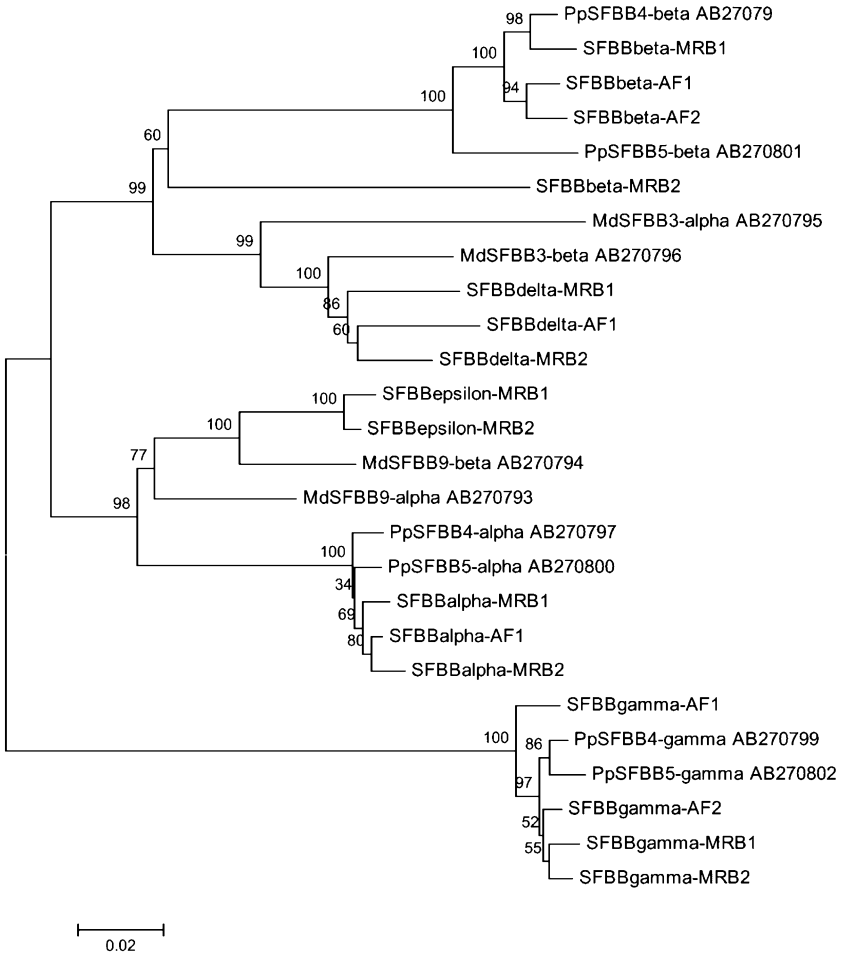

Fig. 1 Neighbor-joining tree of the *SFBB*s identified in the present study and those described by Sassa et al. (2007) (reported with their accession numbers) with 1,000 bootstraps; bootstrap values are reported as percentages

JoinMap 3.0 (Van Ooijen and Voorrips 2002). The graphical representation of Linkage Group 17 was obtained with MapChart 2.0 (Voorrips 2002).

Results

Sequence analysis

The primers developed based on the *SFBB*s reported by Sassa et al. (2007) allowed the amplification of homolog genes from the two European pear cultivars analyzed; 16 polymorphic sequences (Table 2) were obtained, six from "Abbé Fétel" and ten from "Max Red Bartlett," and grouped within the five main clusters corresponding to the genes *SFBBα* to *SFBBε* described in our accompanying article (De Franceschi et al. submitted).

The three primer pairs PpAlpha for/rev, PpBeta for/rev2, and PpGamma for/rev (Table 1) based on *P. pyrifolia SFBBs* sequence allowed the amplification of homologue genes from both cultivars. For the gene *SFBBα*, which was amplified with primers based on the $PpSFBB^{\alpha}$ gene, two different sequences were obtained from "Max Red Bartlett" and only one from "Abbé Fétel." For *SFBBβ* and *SFBBγ*, amplified with primers based on $PpSFBB^{\beta}$ and $PpSFBB^{\gamma}$, respectively, two different sequences were obtained from each cultivar, each putatively belonging to a different S-haplotype. Using the primers designed according to the apple $MdSFBB^{3\text{-}\alpha}$ and $MdSFBB^{3\text{-}\beta}$ genes (primers Md3 for/rev), a single sequence was obtained from "Abbé Fétel" and two from "Max Red Bartlett" (*SFBBδ* genes); finally, the primer pair Md9Beta for/Md9 rev amplified only from "Max Red Bartlett" yielding two different sequences (*SFBBε* gene) homologue to $MdSFBB^{9\text{-}\beta}$. The primer pair Md9Alpha for/Md9 rev designed on the $MdSFBB^{9\text{-}\alpha}$ gene (De Franceschi et al. submitted) was applied but did not produce amplification from any of the two parental varieties.

As expected, the cloned genes exhibited a high homology with the *SFBB*s; the nucleotide p-distances between *SFBB* sequences are reported in Table 3. The identified *SFBBα*, *SFBBβ*, and *SFBBγ* genes from European pear resulted highly homologous to $PpSFBB^{\alpha}$, $PpSFBB^{\beta}$, and $PpSFBB^{\gamma}$, respectively. Thus, the p-distances within the three groups range from 0.011 to 0.058, with the only exception of the *SFBBβ-MRB2* from "Max Red Bartlett" that displays a higher distance. The *SFBBδ* and *SFBBε*

Table 3 Nucleotide p-distances between the European pear *SFBB* genes described in this article and the *SFBB*s from Japanese pear (*PpSFBB*) and apple (*MdSFBB*) reported by Sassa et al. (2007)

	PpSFBB$^{4\text{-}\alpha}$	*PpSFBB*$^{5\text{-}\alpha}$	*PpSFBB*$^{4\text{-}\beta}$	*PpSFBB*$^{5\text{-}\beta}$	*PpSFBB*$^{4\text{-}\gamma}$	*PpSFBB*$^{5\text{-}\gamma}$	*MdSFBB*$^{3\text{-}\alpha}$	*MdSFBB*$^{3\text{-}\beta}$	*MdSFBB*$^{9\text{-}\alpha}$	*MdSFBB*$^{9\text{-}\beta}$
SFBBα-AF1	**0.013**	**0.013**	0.167	0.170	0.189	0.190	0.176	0.149	0.088	0.099
SFBBα-MRB1	**0.016**	**0.013**	0.167	0.171	0.189	0.192	0.179	0.152	0.092	0.103
SFBBα-MRB2	**0.019**	**0.019**	0.171	0.173	0.192	0.196	0.178	0.153	0.093	0.104
SFBBβ-AF1	0.166	0.169	**0.025**	**0.057**	0.229	0.231	0.163	0.149	0.160	0.173
SFBBβ-AF2	0.166	0.169	**0.026**	**0.053**	0.231	0.234	0.166	0.154	0.164	0.173
SFBBβ-MRB1	0.170	0.172	**0.017**	**0.054**	0.229	0.234	0.163	0.151	0.164	0.174
SFBBβ-MRB2	0.164	0.165	0.151	0.163	0.207	0.211	0.156	0.154	0.156	0.164
SFBBγ-AF1	0.189	0.191	0.225	0.226	**0.020**	**0.027**	0.226	0.194	0.170	0.173
SFBBγ-AF2	0.189	0.191	0.224	0.226	**0.011**	**0.015**	0.222	0.190	0.167	0.176
SFBBγ-MRB1	0.190	0.192	0.230	0.229	**0.018**	**0.022**	0.226	0.192	0.173	0.178
SFBBγ-MRB2	0.190	0.192	0.227	0.228	**0.013**	**0.017**	0.225	0.192	0.171	0.178
SFBBδ-AF1	0.152	0.155	0.163	0.163	0.200	0.202	0.115	**0.061**	0.146	0.154
SFBBδ-MRB1	0.148	0.147	0.156	0.156	0.192	0.197	0.117	**0.057**	0.144	0.152
SFBBδ-MRB2	0.144	0.145	0.145	0.147	0.191	0.200	0.104	**0.054**	0.138	0.145
SFBBε-MRB1	0.104	0.102	0.176	0.181	0.185	0.185	0.179	0.155	0.078	**0.058**
SFBBε-MRB2	0.104	0.102	0.173	0.176	0.183	0.183	0.173	0.153	0.075	**0.052**

Values reported in bold underline the homology within the five main clusters corresponding to *SFBB-α* to *ε*

Table 4 CAPS markers realized on the sequence data

Gene	Restriction enzyme	Mapped sequences	Restriction profile (fragment sizes in bp)
SFBBα	*Rsa*I	*SFBBα-AF1*	383-314-258-123-118-45
		SFBBα-MRB1	383-359-258-123-118
		SFBBα-MRB2	742-258-123-118
SFBBβ	*Rsa*I	*SFBBβ-MRB1*	539-322-220-132-57
		SFBBβ-MRB2	713-374-132
SFBBγ	*Mse*I (*Tru*1I)	*SFBBγ-AF1*	351-302-246-129-111-68-39
		SFBBγ-AF2	351-302-246-168-111-68
		SFBBγ-MRB1	351-302-246-150-129-68
		SFBBγ-MRB2	351-302-246-168-111-68
SFBBδ	*Mse*I (*Tru*1I)	*SFBBδ-AF1*	648-165-81-66
		SFBBδ-MRB1	756-165-39
		SFBBδ-MRB2	690-165-66-39
SFBBε	*Eco*RI	*SFBBε-MRB1*	832-452
		SFBBε-MRB2	452-417-415

For each *SFBB* gene, the restriction enzymes used for PCR product digestion, the mapped sequences, and the restriction profile for each sequence are reported

resulted homologous to the $MdSFBB^{3\text{-}\beta}$ and $MdSFBB^{9\text{-}\beta}$ genes, respectively, with p-distances under 0.061 and 0.058 within the two groups.

The neighbor-joining tree of the identified F-box genes and the apple and Japanese pear *SFBB*s (Fig. 1) highlights five well-separated branches, where the European pear *SFBB*s from α to ε are grouped together with the *SFBB*s to which they are homologous.

Segregation analysis and mapping

The identified *SFBB* genes were mapped in a progeny of 92 individuals from the cross "Abbé Fétel" × "Max Red Bartlett." The in silico analysis of the polymorphic restriction sites in the sequences allowed the development of specific CAPS markers (Table 4) for analyzing the segregation of these genes in the population.

The segregation analysis showed that *SFBBδ* and *SFBBε* were fully linked to the *S-RNase* gene: *SFBBδ-AF1* cosegregated with the S_{105} allele, *SFBBδ-MRB1*, and *SFBBε-MRB2* with S_{101}, and *SFBBδ-MRB2* and *SFBBε-MRB1* with S_{102} (Table 5). The genes *SFBBα*, *SFBBβ*, and *SFBBγ*, despite a strong linkage to the S-locus, exhibited very rare recombination events with the *S-RNase*:

- In the *SFBBα* group, besides the *SFBBα-AF1* allele that fully cosegregated with the S_{105}-*RNase*, *SFBBα-MRB1* and *SFBBα-MRB2* cosegregated with S_{101} and S_{102}, respectively, only in 90 of 92 progeny individuals, the remaining two displaying a recombination event between the *S-RNase* and the *SFBBα* gene.
- In the *SFBBβ* group, it was not possible determining the segregation of the "Abbé Fétel" alleles; the bands predicted by the in silico analysis of restriction sites resulted nonpolymorphic or produced distorted frequencies in the population (data not shown); this is probably due to the presence of more than one copy of this gene, with extra copies producing restriction fragments of the same size of those produced by *SFBBβ-AF1* and *SFBBβ-AF2*. The sequences *SFBBβ-MRB1* and *SFBBβ-MRB2* from "Max Red Bartlett," on the contrary, produced at least one restriction fragment that exhibited an 1:1 presence/absence pattern in the progeny, allowing to determine their distribution. *SFBBβ-MRB1* exhibited a full linkage to the S_{101}-*RNase*, whereas *SFBBβ-MRB2* cosegregated with S_{102} in all the individuals except one; the recombinant genotype was one of the two that also resulted recombinant for the *SFBBα* gene. Due to their sequence divergence (Table 3) and their noncomplementary distribution, *SFBBβ-MRB1* and *SFBBβ-MRB2* were mapped as separate genes.
- In the *SFBBγ* group, the alleles *SFBBγ-AF1* and *SFBBγ-AF2* from "Abbé Fétel" cosegregated, respectively, with S_{105} and $S_{104\text{-}2}$-*RNase*s in 91 of 92 cases, highlighting thus the presence of one recombinant; *SFBBγ-MRB1* and *SFBBγ-MRB2* from "Max Red Bartlett" resulted, however fully linked, respectively, to the S_{101} and S_{102} alleles.

In both parents, the S-locus was mapped close to the bottom of LG 17, as previously reported both for apple (Maliepaard et al. 1998) and pear (Yamamoto et al. 2002). The graphical representation of the chromosome is reported in Fig. 2, whereas Table 5 summarizes the linkage of *SFBB*s with each of the four S-haplotypes. The linkage group 17 was unequivocally identified by the presence of some microsatellite markers (CH04C06, CH04C10, CH01H01, AY187627, and Hi07H02), which had been

Table 5 Summary of the *SFBB* genes linked to each of the four S-haplotypes

S-haplotype	Linked F-box genes				
	SFBBα	*SFBBβ*	*SFBBγ*	*SFBBδ*	*SFBBε*
S_{101}	*SFBBα-MRB1*[a]	*SFBBβ-MRB1*	*SFBBγ-MRB1*	*SFBBδ-MRB1*	*SFBBε-MRB2*
S_{102}	*SFBBα-MRB2*[a]	*SFBBβ-MRB2*[b]	*SFBBγ-MRB2*	*SFBBδ-MRB2*	*SFBBε-MRB1*
$S_{104\text{-}2}$			*SFBBγ-AF2*[b]		
S_{105}	*SFBBα-AF1*		*SFBBγ-AF1*[b]	*SFBBδ-AF1*	

[a] Linkage found in 90 of 92 individuals

[b] Linkage found in 91 of 92 individuals

previously mapped in LG 17 of apple and pear (Liebhard et al. 2002, 2003; Yamamoto et al. 2002; Dondini et al. 2004; Patocchi et al. 2005; Silfverberg-Dilworth et al. 2006). The genes *SFBBδ*, *SFBBε*, and *SFBBβ-MRB1* comapped with the *S-RNase*, whereas the other *SFBB*s were placed in its close proximity: *SFBBα* and *SFBBβ-MRB2* in the proximal and *SFBBγ* in the distal region flanking the S-locus.

Discussion

The presence of multiple F-box genes within the S-locus region of plants species with S-RNase-based GSI seems to be a common feature, which has been reported in the genera *Petunia* and *Nicotiana* (Solanaceae), *Antirrhinum* (Plantaginaceae), *Prunus, Malus,* and *Pyrus* (Rosaceae; Lai et al. 2002; Entani et al. 2003; Ushijima et al. 2003; Wang et al. 2004; Sassa et al. 2007; Wheeler and Newbigin 2007). However, in all the species in which the identity of the pollen S factor has been determined experimentally through transgenic approaches (Sijacic et al. 2004; Qiao et al. 2004) or mutant analysis (Ushijima et al. 2004; Sonneveld et al. 2005; Hauck et al. 2006; Tsukamoto et al. 2006; Vilanova et al. 2006), it proved to be a single gene. Therefore, the function of the other *SFB/SLF*-related genes within the S-locus region of these species remains unclear.

In the subtribe Pyrinae of the Rosaceae, the first multiple S-locus F-box genes were characterized by Sassa et al. (2007) and were named *SFBB*s (S-locus F-Box Brothers) due to their multiplicity within S-haplotypes; two *SFBB*s

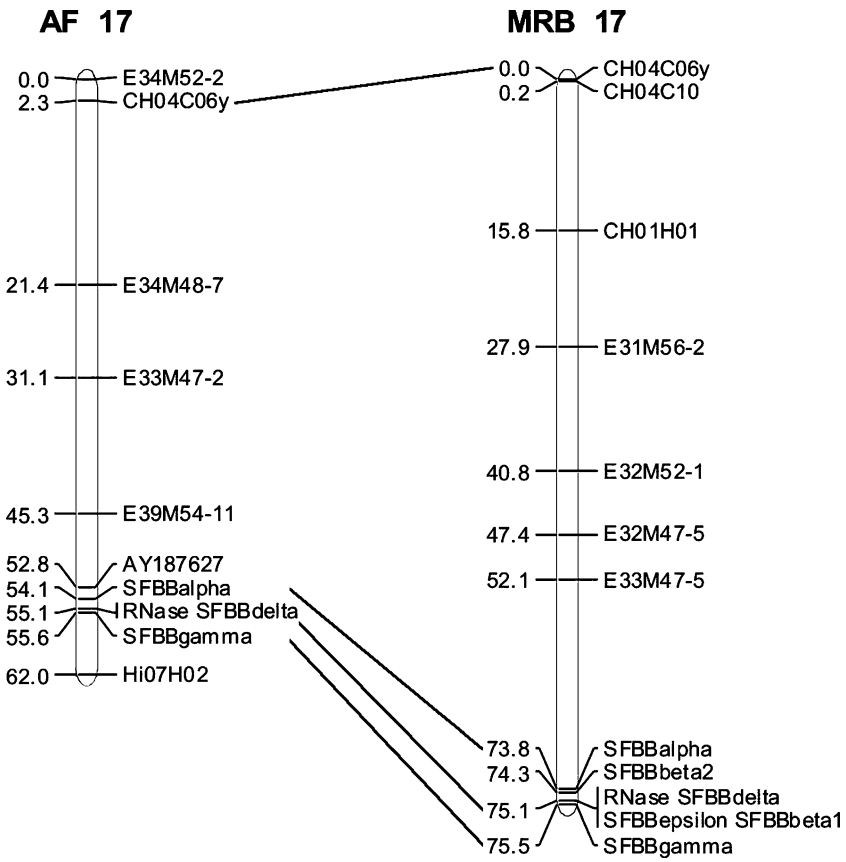

Fig. 2 Graphical representation of the linkage group 17 from the varieties "Abbé Fétel" ("AF," on the left) and "Max Red Bartlett" ("MRB"). As expected, the S-locus was placed on the bottom of the chromosome; the F-box genes *SFBBδ*, *SFBBε*, and *SFBBβ-MRB1* (indicated in the image as "SFBBbeta1") comapped with the *S-RNase*, whereas *SFBBα*, *SFBBγ*, and *SFBBβ-MRB2* (indicated as "SFBBbeta2") were placed in its close proximity

were identified in S-haplotypes of apple, whereas three *SFBB*s were identified in S-haplotypes of Japanese pear. Our previous findings (De Franceschi et al. submitted) suggested that the different *Pyrinae* species share a common S-locus structure, having F-box genes homologous to the Japanese pear and apple *SFBB*s. In the present work, evidence is provided showing their tight linkage to the S-locus. This highlights that the number of F-box genes surrounding the *S-RNase* must be higher than initially expected; despite the two genes reported in apple and three in Japanese pear, up to five different F-box genes have been cloned and proved to be linked to the *S-RNase*; it should, moreover, be considered that the primer pair developed on $PpSFBB^{\beta}$ most likely amplified two different genes (*SFBBβ-MRB1* and *SFBBβ-MRB2*), both resulting S-linked, even though in different measures. For two of the four analyzed S-haplotypes (S_{101} and $S_{104\text{-}2}$), another linked F-box gene has recently been reported by Zisovich et al. (2009). Altogether these results suggest that up to eight different *SFBB* genes might be associated to the S-locus of the Pyrinae, but this should be still considered a provisional estimate; further information on this regard will hopefully rise from genome sequencing projects. In a recent review, Sassa et al. (2010) anticipated the existence of up to ten different *SFBB*-related genes in species of *Pyrinae*, which supports this possibility.

The role of these multiple F-box genes surrounding the S-RNase is an aspect that still needs to be elucidated. Since the *SFBBs* identified by Sassa et al. (2007) resulted specifically expressed in pollen, they are supposed to exert their function during pollen tube growth; an expression analysis was not carried out for the European pear *SFBBs* hereby described, but due to their high homology with the apple and Japanese pear ones, it seems reasonable hypothesizing that they exhibit the same pollen-specific expression. Their high number makes unlikely that all of them act together in the determination of pollen S specificity, as it was initially proposed, but they might somehow contribute to pollen viability and fitness.

In the present study, moreover, we used for the first time a mapping approach to estimate the actual extent of the genetic linkage between the identified F-box genes and the *S-RNase*. Each gene was placed on a molecular map, developed on a population of 92 individuals from the cross "Abbé Fétel" × "Max Red Bartlett." Some of the genes (*SFBBδ*, *SFBBε*, and *SFBBβ-MRB1*) resulted fully linked to the S-locus, whereas others (*SFBBα*, *SFBBβ-MRB2*, and *SFBBγ*) evidenced a weaker linkage, allowing the identification of very rare recombinant genotypes. It is worth noting that *SFBBδ* and *SFBBε* are homologous to the apple *SFBB*s, which were identified by Sassa et al. (2007) from BAC clones of a genomic library, i.e., physically close to the *S-RNase*. The observation that *SFBBδ* and *SFBBε* displayed no recombination events with the *S-RNase* supports the assumption that these two genes, like their apple homologs, are in close proximity to the *S-RNase*. On the contrary, *SFBBα*, *SFBBβ*, and *SFBBγ* are homologous to the Japanese pear *SFBB*s, which were identified from pollen cDNA, and for which information was not available on their physical position within the S-locus.

Even though a precise determination of the linkage between each *SFBB* and the *S-RNase* would require a fine-mapping approach involving a much larger population, the segregation analysis on 92 individuals allowed us to unequivocally conclude that not all the F-box genes placed in the region surrounding the *S-RNase* actually belong to the S-locus. The evidence of recombination between the *S-RNase* gene and *SFBBα*, *SFBBβ-MRB2* and *SFBBγ* excludes these genes from the role of candidates for the pollen S determinant; recombination between the female and male determinant of GSI has never been described for S-RNase-based self-incompatibility; moreover, such a rearrangement would result in haltered S-haplotypes, with different pistil and pollen S specificities, which theoretically would confer self-compatibility due to the misrecognition of "self" pollen. However, all the recombinant genotypes exhibited a normal self-incompatible behavior (data not shown). These data provide an experimental support for the hypothesis recently suggested by Vieira et al. (2009), which, after an analysis of positively selected amino acid sites in the $PpSFBB^{\gamma}$ genes, found very unlikely an involvement of this gene in S-specificity determination and an effect of the S-locus on its evolution patterns.

The weaker linkage of *SFBBα* and *SFBBγ* with the *S-RNase* also provides an explanation for the different patterns of sequence similarity observed among apple and Japanese pear *SFBB*s. The high level of sequence diversity of the *S-RNase* gene is believed to be the result of selection favoring amino acid replacement, causing the creation of new S specificities (Ioerger et al. 1991; Ishimizu et al. 1998; Takebayashi et al. 2003; Igic et al. 2007; Vieira et al. 2007). The same mechanism might also affect the F-box genes belonging to the S-locus that are involved in determining the pollen S function (Ikeda et al. 2004; Nunes et al. 2006), even though it is not clear in which measure it should happen (Newbigin et al. 2008; De Franceschi et al. submitted). The genes *SFBBα* and *SFBBγ*, corresponding to $PpSFBB^{\alpha}$ and $PpSFBB^{\gamma}$, proved to be tightly linked, but not belonging, to the S-locus. For this reason, they could have escaped this kind of selection affecting the S-locus genes and generated by the long-term evolution under frequency-dependent balancing selection; they might thus have evolved as linked, but separated loci.

The evidence of multiple F-box genes belonging or tightly linked to the S-locus in European pear might contribute to elucidating the GSI mechanism in the Pyrinae, but it raises as well a series of questions yet to be solved.

First, the actual number of S-locus F-box genes remains unknown; even if it proved to be higher than expected, it is difficult to hypothesize how many of them have yet to be discovered. Moreover, it must be considered that not all the S-haplotypes seem to carry the same F-box genes in the same positions: the evidence of gene duplication (as described for *SFBBβ*) and the lack of amplification of some genes from some haplotypes (as for *SFBBα*, *SFBBδ*, and *SFBBε*) suggest that different S-haplotypes might differentiate from each other even under this aspect.

Finally, the main question to which a clear answer has yet to be provided is the nature of the pollen S factor in the Pyrinae. The high number of F-box genes might be a distinctive feature of the S-locus in this subtribe, but the role played by these genes remains unclear (Sassa et al. 2010). It is possible that, like in the other taxa in which the identity of pollen S was determined, it depends on a single F-box gene, but it cannot be excluded that more than one gene participate to this function.

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