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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

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Recently, an *S* haplotype–specific F-box (*SFB*) gene has been proposed as a candidate for the pollen-*S* specificity gene of RNase-mediated gametophytic self-incompatibility in Prunus (Rosaceae). We have examined two pollen-part mutant haplotypes of sweet cherry (*Prunus avium*). Both were found to retain the *S-RNase*, which determines stylar specificity, but one (S_3' in JI 2434) has a deletion including the haplotype-specific *SFB* gene, and the other (S_4' in JI 2420) has a frame-shift mutation of the haplotype-specific *SFB* gene, causing amino acid substitutions and premature termination of the protein. The loss or significant alteration of this highly polymorphic gene and the concomitant loss of pollen self-incompatibility function provides compelling evidence that the *SFB* gene encodes the pollen specificity component of self-incompatibility in Prunus. These loss-of-function mutations are inconsistent with SFB being the inactivator of non-self *S*-RNases and indicate the presence of a general inactivation mechanism, with SFB conferring specificity by protecting self *S*-RNases from inactivation.

INTRODUCTION

Self-incompatibility (SI) prevents inbreeding in a wide range of flowering plants (reviewed in De Nettancourt, 2001). The mechanism can be either gametophytic or sporophytic depending on the genetic control of the pollen SI phenotype. In sweet cherry (*Prunus avium*), a member of the Rosaceae cultivated for its fruit, SI and cross-incompatibility between various pairs of cultivars was attributed to a multiallelic *S* locus, expressed gametophytically (Crane and Lawrence, 1929). As in all other SI systems studied, the *S* locus of cherry is considered to comprise at least two parts, one expressed in the style and a complementary one expressed in the pollen, as originally proposed by Lewis (1949).

Initially the sporophytic SI of Brassica and the gametophytic SI of the Solanaceae were the most intensively studied at the molecular level, but recently several important breakthroughs have been made in the Rosaceae, which appears to have the same SI mechanism as the Solanaceae. The molecular identification of the pistil components of SI has revealed three distinct mechanisms in the sporophytic SI system of Brassica and in the gametophytic SI systems of the Solanaceae/Rosaceae/Scrophulariaceae and of Papaver (De Nettancourt, 2001). In the Solanaceae, the Rosaceae, and the Scrophulariaceae, the stylar part codes for a ribonuclease (*S*-RNase) (McClure et al., 1989; Broothaerts et al., 1995; Bošković and Tobutt, 1996; Xue et al., 1996). Ribonuclease activity of *S*-RNases is needed to inhibit the growth of pollen tubes carrying an *S* allele that matches an *S* allele present in the style (Huang et al., 1994; Royo et al., 1994), supporting a model for this type of gametophytic SI in which pollen RNA is degraded in an incompatible interaction.

The pollen component (pollen-*S*) in Brassica has been identified as a ligand for a receptor kinase located in the stigma (reviewed in Brugière et al., 2000). Candidates for the pollen-*S* gene of RNase-based SI have recently been identified in Antirrhinum of the Scrophulariaceae (Lai et al., 2002), Prunus (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003), and Petunia of the Solanaceae (Qiao et al., 2004; Sijacic et al., 2004). These are all *S*-linked F-box genes (*SLF* or *SFB*) and have implicated ubiquitination in the SI reaction. In this article, we follow the use of the abbreviation SFB (for *S* haplotype–specific F-box) in cherry (Yamane et al., 2003). Additional F-box genes (*SLFL1-3*) have been identified in Prunus *S* haplotypes, although these exhibit relatively low levels of polymorphism (Entani et al., 2003; Ushijima et al., 2003).

Two self-compatible cherry selections raised at the John Innes Institute (Lewis and Crowe, 1954) offer a unique opportunity for studying the molecular basis of loss of pollen-*S* function at a rosaceous *S* locus. Gain-of-function or loss-of-function transgenic experiments in Prunus tree species are hindered by the lack of an efficient transformation system and a long juvenile period. The self-compatible selections, JI 2420 and JI 2434, were obtained from the nominally incompatible cross of the cultivars Emperor Francis (S_3S_4) × Napoleon (S_3S_4, X-irradiated pollen). Both selections carry pollen-part mutations but retain normal

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Table 1. Erika (S_1S_3) × Lapins (S_1S_4') Expected Gamete Genotypes and Seedling Genotypes (and *S*-RNase Phenotypes) if S_4' Results from a Duplication of Pollen-S_3 (dS_3) Not Linked in Coupling with S_4

Female/Male	S_1	S_4	S_1dS_3	S_4dS_3
S_1	X	S_1S_4 (S_1S_4)	S_1S_1dS_3 (S_1)	S_1S_4dS_3 (S_1S_4)
S_3	X	S_3S_4 (S_3S_4)	S_1S_3dS_3 (S_1S_3)	S_3S_4dS_3 (S_3S_4)

X indicates incompatibility of pollen.

stylar function (Matthews and Lapins, 1967; Matthews, 1970). Recently, the genotypes of JI 2420 and two accessions of JI 2434 were clarified, and the basis of their self-compatibility was studied using stylar ribonuclease analyses and test crosses (Bošković et al., 2000). In JI 2420, self-compatibility is attributed to a mutant S_4 allele, denoted S_4', where the prime symbol indicates the loss of pollen-*S* function (Lewis and Crowe, 1954). Similarly, in JI 2434, the self-compatibility is attributed to a mutant S_3 allele, S_3'.

Self-compatible pollen-part mutants obtained by selfing or by crossing nominally incompatible plants using x-irradiated pollen have been described not only in *P. avium*, but also in *Oenothera organensis* of the Onagraceae (Lewis, 1949, 1951), *Petunia inflata* (Brewbaker and Shapiro, 1959), and *Nicotiana alata* (Pandey, 1965). Brewbaker and Natarajan (1960) found that all pollen-part mutants studied in *P. inflata* carried a centric fragment. Centric fragments, which can result from x-irradiation, are parts of a chromosome including the centromere that segregate randomly at meiosis. The centric fragments in the *P. inflata* pollen-part mutants carried a duplicated *S* allele, resulting in some pollen grains being heteroallelic. The self-compatibility of heteroallelic pollen had been advanced by Lewis and Modlibowska (1942) to explain the self-compatibility of a tetraploid pear (*Pyrus communis*)—pollen grains of genotype S_1S_2 succeed on $S_1S_1S_2S_2$ styles, whereas S_1S_1 and S_2S_2 pollen fails. The cross of the tetraploid as pollen parent on to the original diploid succeeds, whereas the reciprocal cross fails, indicating a breakdown of pollen-part function. This interaction between the two *S* alleles in heteroallelic pollen has been termed competitive interaction (Lewis, 1943). Brewbaker and Natarajan (1960) also concluded that the pollen-part mutants of Oenothera and Prunus, studied by Lewis, could have resulted from a duplication of an *S* allele. However, Lewis (1961) concluded that some pollen-part mutations of Oenothera should be the result of a mutation or deletion of the pollen part and stated that most of his arguments were also valid for the pollen-part mutant selections of Prunus.

In recent years, molecular analyses have been performed on pollen-part mutants generated by irradiation mutagenesis in *Solanum tuberosum* (Thompson et al., 1991) and *N. alata* (Golz et al., 1999, 2001) of the Solanaceae. In both studies, DNA gel blot analysis with *S-RNase* probes showed that some of the self-compatible mutants obtained had an extra *S* allele, either carried on a centric fragment or translocated to another chromosome. In some cases, no additional *S-RNase* allele was detected, although there was evidence for a chromosomal duplication, presumably including the pollen part but not the *S-RNase* (Golz et al., 2001). No evidence of a deletion was found in either study.

The cherry pollen-part mutations in JI 2420 and JI 2434 could, we surmised, have resulted from duplication of a pollen-*S* gene giving heteroallelic pollen or from a mutation in the pollen-*S* gene. Thus, the S_3' mutant allele of JI 2434 could either be a result of a duplicate S_4 pollen-*S* allele (dS_4) giving rise to S_3dS_4 pollen that would be compatible on an S_3S_4 style or else a mutation of the S_3 pollen-*S* gene itself. Similarly, explanations of self-compatibility in the S_4' mutant JI 2420 are a duplicate S_3 pollen-*S* allele (dS_3) resulting in S_4dS_3 pollen or a mutation of the S_4 pollen-*S* gene itself. Bošković et al. (2000) found no evidence for a duplicated *S-RNase* in either mutant selection with ribonuclease activity assays.

A molecular analysis of these cherry mutant *S* alleles has now been made possible after the cDNA cloning of the *S-RNases* for the S_3- and S_4-haplotypes (Sonneveld et al., 2001). We demonstrate that there is no evidence of a duplication in either mutant haplotype, but rather a deletion or mutation affecting the haplotype-specific *SFB* gene, leading to loss of function. These findings provide further evidence for the pollen-*S* function of *SFB* genes, and we discuss how these unique loss-of-function mutations can be reconciled with current models of RNase-based SI.

RESULTS

Genetic Analysis of Pollen-Part Mutations

Pollen-part mutations can theoretically result from duplication of a pollen-*S* allele or from a mutation of the pollen-*S* gene itself. Heteroallelic pollen can arise when a duplicated pollen-*S* gene has been translocated to the chromosome bearing the other *S* allele, or to a non-*S* chromosome, or it could be carried on a centric fragment. In the first case, the pollen-part mutation will be linked in coupling to one particular *S* allele; in the other two cases, the pollen-part mutation will segregate independently from the *S* locus. A mutation in the pollen part itself would always be associated with one *S* allele.

To confirm that the pollen-part mutation in JI 2420 is linked in coupling with S_4, the progeny of a cross between a pollen parent deriving its self-compatibility from JI 2420 but not having S_3 (Lapins, S_1S_4') and a female parent with the S_1 allele in common (Erika, S_1S_3) was analyzed. If the mutation was not linked with S_4 (i.e., in the case of a duplication of the S_3 pollen-*S* allele elsewhere in the genome), both types of pollen (S_1 and S_4), when carrying the duplicated S_3 pollen-*S* allele, would be

Table 2. Erika (S_1S_3) × Lapins (S_1S_4') Expected Gamete Genotypes and Seedling Genotypes (and *S*-RNase Phenotypes) if S_4' Results from a Deletion/Mutation of the S_4 Pollen-*S* Allele (for Convenience Indicated by S_4') or from a Duplication of Pollen-S_3 (dS_3) Linked in Coupling with S_4

Female/Male	S_1	S_4' or S_4dS_3
S_1	X	S_1S_4' or S_1S_4dS_3 (S_1S_4)
S_3	X	S_3S_4' or S_3S_4dS_3 (S_3S_4)

X indicates incompatibility of pollen.

heteroallelic and able to grow on the style of the female parent even if it has the S_1 allele in common (Tables 1 and 2).

Analysis of stylar ribonucleases showed that 23 seedlings had bands for S_1 and S_4 (S_1S_4' seedlings), and 18 seedlings had bands for S_3 and S_4 (S_3S_4' seedlings) (data not shown). None of the seedlings showed a single band in the S_1 position. Consistent rejection of the S_1 pollen confirms that the mutation of JI 2420 is linked in coupling with S_4 and cannot be attributed to a duplication of the S_3 pollen-*S* allele on a centric fragment or on a non-*S* chromosome.

Likewise, to confirm that self-compatibility in JI 2434 ($S_3'S_4$) is linked in coupling with S_3, a progeny from a cross between a pollen parent deriving self-compatibility from JI 2434 but not having the S_4 allele (9239-3, S_1S_3') and a female parent with the S_1 allele in common (Van, S_1S_3) was raised and analyzed. The presence or absence of S_1S_1' genotypes (single S_1 band) in the progeny would indicate whether self-compatibility of JI 2434 is linked with S_3 (Tables 3 and 4).

S allele genotyping of 46 seedlings by PCR using consensus primers for the first intron of cherry *S-RNases* showed that they segregated into two classes (example shown in Figure 1): 17 with bands for S_3 and S_1 (S_1S_3' seedlings) and 29 with a single S_3 band (S_3S_3' seedlings). The absence of a class with a single S_1 band (S_1S_1' seedlings) indicates that the pollen-part mutation of JI 2434 ($S_3'S_4$) is linked in coupling with S_3 and cannot be attributable to a duplication of the S_4 pollen component on a centric fragment or on a non-*S* chromosome.

DNA Gel Blot Analysis of *S-RNase* Regions in Pollen-Part Mutants

Because both pollen-part mutant selections appeared to have the mutation linked with a particular *S* allele, DNA gel blot analysis with an *S-RNase* probe was performed to look for evidence of a rearrangement in the regions flanking the appropriate *S-RNases*.

Digestion of genomic DNA with both restriction enzymes *Eco*RI (Figure 2A) and *Hin*dIII (Figure 2B) resulted in a single restriction fragment in the normal S_4 position for JI 2420 (S_4S_4') (i.e., the S_4' band was indistinguishable from S_4). In the *Hin*dIII digest, the S_3' restriction fragment of JI 2434 was in the same position as the S_3 fragment from the parents and the standard Van (S_1S_3). In the *Eco*RI digest, however, the S_3' restriction fragment of JI 2434 appeared to be slightly smaller than the S_3 fragment. This indicated an alteration in the region flanking the S_3-*RNase* of JI 2434.

Table 3. Van (S_1S_3) × 9239-3 (S_1S_3') Expected Gamete Genotypes and Seedling Genotypes (and *S*-RNase Phenotypes) if S_3' Results from a Duplication of Pollen-S_4 (dS_4) Not Linked in Coupling with S_3

Female/Male	S_1	S_3	S_1dS_4	S_3dS_4
S_1	X	X	S_1S_1dS_4 (S_1)	S_1S_3dS_4 (S_1S_3)
S_3	X	X	S_1S_3dS_4 (S_1S_3)	S_3S_3dS_4 (S_3)

X indicates incompatibility of pollen.

Table 4. Van (S_1S_3) × 9239-3 (S_1S_3') Expected Gamete Genotypes and Seedling Genotypes (and *S*-RNase Phenotypes) if S_3' Results from a Deletion/Mutation of the S_3 Pollen-*S* Allele (for Convenience Indicated by S_3') or from a Duplication of Pollen-S_4 (dS_4) Linked in Coupling with S_3

Female/Male	S_1	S_3' or S_3dS_4
S_1	X	S_1S_3' or S_1S_3dS_4 (S_1S_3)
S_3	X	S_3S_3' or S_3S_3dS_4 (S_3)

X indicates incompatibility of pollen.

Testing four previously genotyped selections from the cross Van (S_1S_3) × JI 2434 ($S_3'S_4$) confirmed the shift of the S_3' restriction fragment in the *Eco*RI digest. As shown in Figure 2C, the S_3S_3' selection shows two bands, confirming that the S_3' fragment has a lower molecular weight than the normal S_3 fragment, indicating a sequence change in the region flanking the S_3-*RNase* of JI 2434. The estimated sizes of the S_3 and the S_3' fragments are ~13 and 11 kb, respectively.

When 11 more restriction enzymes were tested and fragment sizes estimated (Table 5), none of the enzymes showed a fragment size shift for S_4' compared with S_4. A band shift for S_3' was, however, found with *Kpn*I, *Pae*I, *Pst*I, *Sst*I, and *Xba*I, indicating a major sequence rearrangement flanking the S_3-*RNase*. Whether the rearrangement in the S_3'-haplotype is in the 5′ or 3′ flanking region of the S_3-*RNase* was investigated by inclusion of an enzyme cutting at the 5′ end of the S_3-*RNase* sequence (*Pst*I) and an enzyme cutting toward the 3′ end of the S_3-*RNase* sequence (*Bam*HI). Because a band shift was found for *Pst*I, but not for *Bam*HI, it appeared that the rearrangement had occurred downstream of the S_3-*RNase* gene. The *Sst*I digest (Figure 2D) shows that the breakpoint of the rearrangement in the S_3'-haplotype must be within ~3.5 kb of the S_3-*RNase* gene.

The DNA gel blot analyses of the S_1S_3' and the S_3S_3' selection confirmed that JI 2434 does not have a duplicated S_4-*RNase* gene, in accordance with previous reports (Bošković et al., 2000). Similarly, there is no evidence of a duplicated S_3-*RNase* gene in JI 2420.

Cloning of the S_4-Haplotype–Specific *SFB* Sequence

To study the haplotype-specific *SFB* genes, recently identified as candidates for the pollen-*S* gene in Prunus (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003), the S_4-haplotype–specific *SFB* (S_4-*SFB*) genomic sequence was cloned by genomic PCR using degenerate primers. The S_3-haplotype–specific *SFB* (S_3-*SFB*) sequence was available from the EMBL database (Yamane et al., 2003).

The deduced amino acid sequence of the putative S_4-*SFB* was aligned with other Prunus *SFB* sequences (Figure 3). It shows high sequence similarity with the other sequences (74.7 to 81.5% amino acid identity) and contains the expected F-box motif and two variable regions toward the 3′ end (Ushijima et al., 2003).

Cosegregation of the cloned putative S_4-*SFB* sequence with the S_4 allele was confirmed in a progeny segregating for S_4 using allele-specific primers (Figure 4). Seedlings showing

amplification had previously been found to carry the S_4 allele by stylar ribonuclease activity assays (Bošković et al., 1997) and *S-RNase* allele-specific PCR (Sonneveld et al., 2001).

S-RNase and *SFB* Allele-Specific PCR

To check for duplications, deletions, or other rearrangements in the pollen-part mutant selections, allele-specific PCR was performed with S_3- and S_4-specific primers for both *S-RNase* and *SFB* sequences. Several conclusions can be drawn from the allele-specific PCR amplifications shown in Figure 5 and summarized in Table 6.

Amplification with the *S-RNase* primers for parents, standards, and pollen-part mutant selections is consistent with their stylar SI response and with the DNA gel blot analysis described above (Figures 5B and 5D).

Amplification with the *SFB* primers is as expected for parents and standards. For PCR amplification with the *SFB* primers for the S_4' mutant, the results of the homozygous $S_4'S_4'$ selection are informative (Figures 5A and 5C). The selection clearly shows a band for S_4-*SFB* but not for S_3-*SFB*. This indicates that, if *SFB* is the pollen-*S* gene, self-compatibility in the S_4' mutant cannot be attributed to a duplication of the S_3 pollen-*S* allele. This leaves a mutation of the S_4 pollen-*S* allele as the most likely explanation for the pollen-part mutation. If this is the case, the mutation clearly does not affect PCR amplification and does not visibly alter the product size because the S_4' PCR product is indistinguishable on the gel from the product for the S_4 allele.

For the S_3' mutant, again the results of the homozygous selection, $S_3'S_3'$, are informative (Figures 5A and 5C). This shows no band for the S_3- or the S_4-*SFB*, indicating that if *SFB* is the pollen-*S* gene, the S_3' mutation is not a duplication of the S_4 pollen-*S* allele but a mutation of the S_3 pollen-*S* allele itself. The nature of the mutation cannot be deduced from these results, but it must affect at least one of the primer binding sites of the S_3-*SFB* sequence or otherwise interfere with PCR amplification.

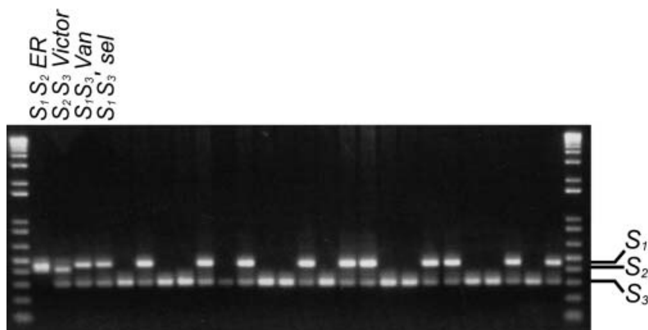

Figure 1. Segregation Analysis of *S* Alleles to Test Cosegregation of the JI 2434 Pollen-Part Mutation with the S_3 Allele.

Genomic DNA of seedlings of Van (S_1S_3) × 9239-3 (S_1S_3') was amplified by PCR using consensus primers revealing length polymorphism of the first intron of cherry *S-RNases*. Samples are as follows: ER, Early Rivers (S_1S_2); Victor (S_2S_3) (standards for S_1 and S_3, respectively); Van (S_1S_3); S_1S_3' selection, 22 seedlings.

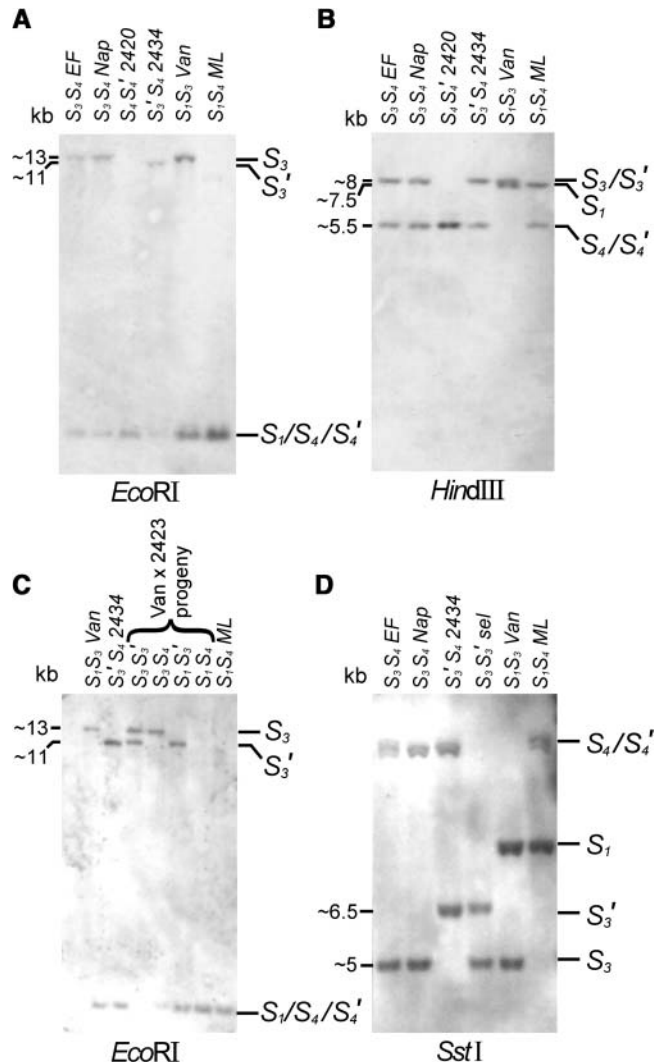

Figure 2. DNA Gel Blot Analysis of the S_3' and S_4' Mutants Using an S_1-*RNase* cDNA Probe to Test for Rearrangements in the Regions Flanking the S_3- and S_4-*RNase* Genes, Respectively.

(A) *Eco*RI digest of parents and pollen-part mutants. EF, Emperor Francis (S_3S_4); Nap, Napoleon (S_3S_4); 2420, JI 2420 (S_4S_4'); 2434, JI 2434 ($S_3'S_4$); Van (S_1S_3); ML, Merton Late (S_1S_4).

(B) *Hin*dIII digest of parents and pollen-part mutants. EF, Emperor Francis (S_3S_4); Nap, Napoleon (S_3S_4); 2420, JI 2420 (S_4S_4'); 2434, JI 2434 ($S_3'S_4$); Van (S_1S_3); ML, Merton Late (S_1S_4).

(C) *Eco*RI digest of the progeny Van (S_1S_3) × JI 2434 ($S_3'S_4$). Van (S_1S_3); 2434, JI 2434 ($S_3'S_4$); S_3S_3' selection; S_3S_4 selection; S_1S_3' selection; S_1S_4 selection; ML, Merton Late (S_1S_4).

(D) *Sst*I digest of parents and S_3' mutant. EF, Emperor Francis (S_3S_4); Nap, Napoleon (S_3S_4); 2434, JI 2434 ($S_3'S_4$); S_3S_3' selection; Van (S_1S_3); ML, Merton Late (S_1S_4).

Low stringency post-hybridization washes allowed the detection of cross-hybridization of the S_1-*RNase* cDNA probe to both the S_3 and S_4 alleles.

Table 5. Restriction Enzymes Used for DNA Gel Blot Analysis with *S-RNase* Probe and Estimated Fragment Sizes for Each of the *S* Alleles Present

	Estimated Size of Restriction Fragments (kb)[a]													
Allele	*Bam*HI[b]	*Bcl*I	*Eco*RI[c]	*Hin*dIII	*Kpn*I[c,d]	*Pst*I[c,e]	*Nco*I	*Nde*I	*Pae*I[c]	*Sca*I[d]	*Sst*I[c]	*Ssp*I[d]	*Xba*I[c]	*Eco*RI/*Hin*dIII
S_1	14.5	7.5	~2	7.5	21.0	23	14.5	6.5	11.5	5.5	11.5	~2	12	~2.0
S_3	9.5	6.0	13	8.0	20.0	7	8.0	9.5	7.5	~4.0	5.0	~3 + 3.5	22	7.5
S_3'	9.5	6.0	11	8.0	6.5	15	8.0	9.5	16.5	~4.0	6.5	~3 + 3.5	20	7.5
S_4	>30.0	7.5	~2	5.5	10.5	~3	4.5	17.0	13.0	6.5	12.5	~1	8	~2.0
S_4'	>30.0	7.5	~2	5.5	10.5	~3	4.5	17.0	13.0	6.5	12.5	~1	8	~2.0

[a] Sizes of the bands were estimated by comparison with the 5-kb DNA ladder (Invitrogen). Please note that the size fragments under 5 kb and over 30 kb could not be estimated accurately.
[b] Restriction site at 3′ end of S_3-*RNase* (just before C5).
[c] Restriction enzyme reveals a fragment size shift for S_3'.
[d] Restriction site in S_3-*RNase* between C3 and RC4 (*Ssp*I), between C2 and C3 (*Kpn*I), and just after RC4 (*Sca*I); the probe does not always detect both resulting fragments.
[e] Restriction site at 5′ end of S_3-*RNase* (just after C1).

DNA Gel Blot Analysis with *SFB* Probes

DNA gel blot analyses with S_3- and S_4-*SFB* probes were performed to confirm the PCR results described above and to characterize the pollen-part mutant haplotypes further. This approach should indicate whether most of the S_3-*SFB* sequence is missing in the S_3' mutant. In addition, it could also check for rearrangements in the regions flanking the S_4-*SFB* gene in the S_4' mutant.

The results of the DNA gel blot analysis with specific *SFB* probes are shown in Figure 6. Each of the parents shows a restriction fragment for the S_3- and the S_4-*SFB* sequence (Figures 6A and 6B). The S_4-*SFB* probe also weakly hybridizes to the S_1 allele of the standards (Figure 6A). With the S_4-*SFB* probe (Figure 6A), the homozygous $S_4'S_4'$ selection shows a restriction fragment in the same position as the parents and standards. Therefore, with the restriction enzyme used (*Dra*I), there is no evidence of a rearrangement in the S_4'-haplotype. Probing with the S_3-*SFB* probe (Figure 6B) reveals that the S_3-*SFB* sequence, at least that included in the probe, is entirely missing in the S_3' mutant, suggesting that the pollen-part mutation involves a deletion of most of the S_3-*SFB* sequence. Sequencing of the probe confirmed that it represents the S_3-*SFB* sequence because it matched the S_3-*SFB* sequence in the database, which has also been shown to give haplotype-specific hybridization signals in DNA gel blot analysis (Yamane et al., 2003). These DNA gel blot data are consistent with the genomic PCR data in Figure 5.

Structure of the S_3- and S_3'-Haplotypes

The result of an attempt to amplify the region between the *S-RNase* and the *SFB* gene in the S_3-haplotype is shown in Figure 7. Allele-specific primers for each of the genes were used in all four possible combinations (Figures 7A to 7D). Only the PCR that included the S_3-*RNase* forward and the S_3-*SFB* forward primers (Figure 7A) gave a product, of ~8 kb, exclusively in samples containing the S_3 allele. The result of the S_3 intergenic PCR reveals the relative orientation of the genes, as well as the distance between them: the S_3-*SFB* gene is located ~6.5 kb downstream of the S_3-*RNase* gene, in opposite transcriptional orientation as shown in Figure 8. This is consistent with the detection of a rearrangement in the S_3'-haplotype downstream of the S_3-*RNase* gene (Table 5) that results in loss of the S_3-*SFB* gene. A similar attempt in the S_4-haplotype failed, presumably because the distance between the two genes in the S_4-haplotype is too great to be amplified under the PCR conditions used.

A preliminary restriction map of the S_3- and S_3'-haplotypes, of Napoleon (S_3S_4) and JI 2434 ($S_3'S_4$), respectively, is shown in Figure 8. The restriction enzyme sites flanking the *S-RNase* genes are based on the DNA gel blot analysis (Table 5). A major sequence rearrangement downstream of the S_3-*RNase* in the S_3'-haplotype includes a deletion of the S_3-*SFB* sequence. The breakpoint of the rearrangement is ~3.5 kb from the S_3-*RNase*.

We examined whether other *S* locus F-box genes (*SLFL*s) are affected by the rearrangement in the S_3'-haplotype. Three *SLFL* genes (*SLFL1-3*) have been identified in two haplotypes of *P. mume*, and the relative gene order (also in relation to *S-RNase* and *SFB*) was found to be conserved (Entani et al., 2003). In these two haplotypes, only *SLFL2* is located downstream of the *S-RNase* and upstream of *SFB*. If the gene order at the Prunus *S* locus is conserved, *SLFL2* is therefore most likely to be affected by the rearrangement in the S_3'-haplotype. *SLFL2*-specific primers amplified a product in Napoleon and in the $S_3'S_3'$ homozygous selection, and sequencing of the product showed that they were almost identical. The sequence is ~96% identical to the two *P. mume SLFL2* sequences and ~67% identical to other Prunus *SLFLs*, indicating that the *SLFL2* gene is not deleted in the S_3'-haplotype.

The *SLFL2* product was used as a probe in DNA gel blot analysis. Two genomic digests, *Dra*I and *Sst*I, confirmed the presence of an *SLFL2* fragment in the S_3'-haplotype. The *Dra*I digest gave a monomorphic band of ~2 kb (data not shown), but the *Sst*I digest revealed limited haplotype-specific polymorphism (Figure 9). The S_3' fragment is approximately the same size as that of S_3, indicating that the genomic region around the *SLFL2* gene is not rearranged (Figure 9).

Cloning of the S_4'-Haplotype–Specific *SFB*

If *SFB* is the pollen-*S* gene in cherry, a mutation affecting the S_4-*SFB* is expected because no duplication of the S_3-*SFB* was

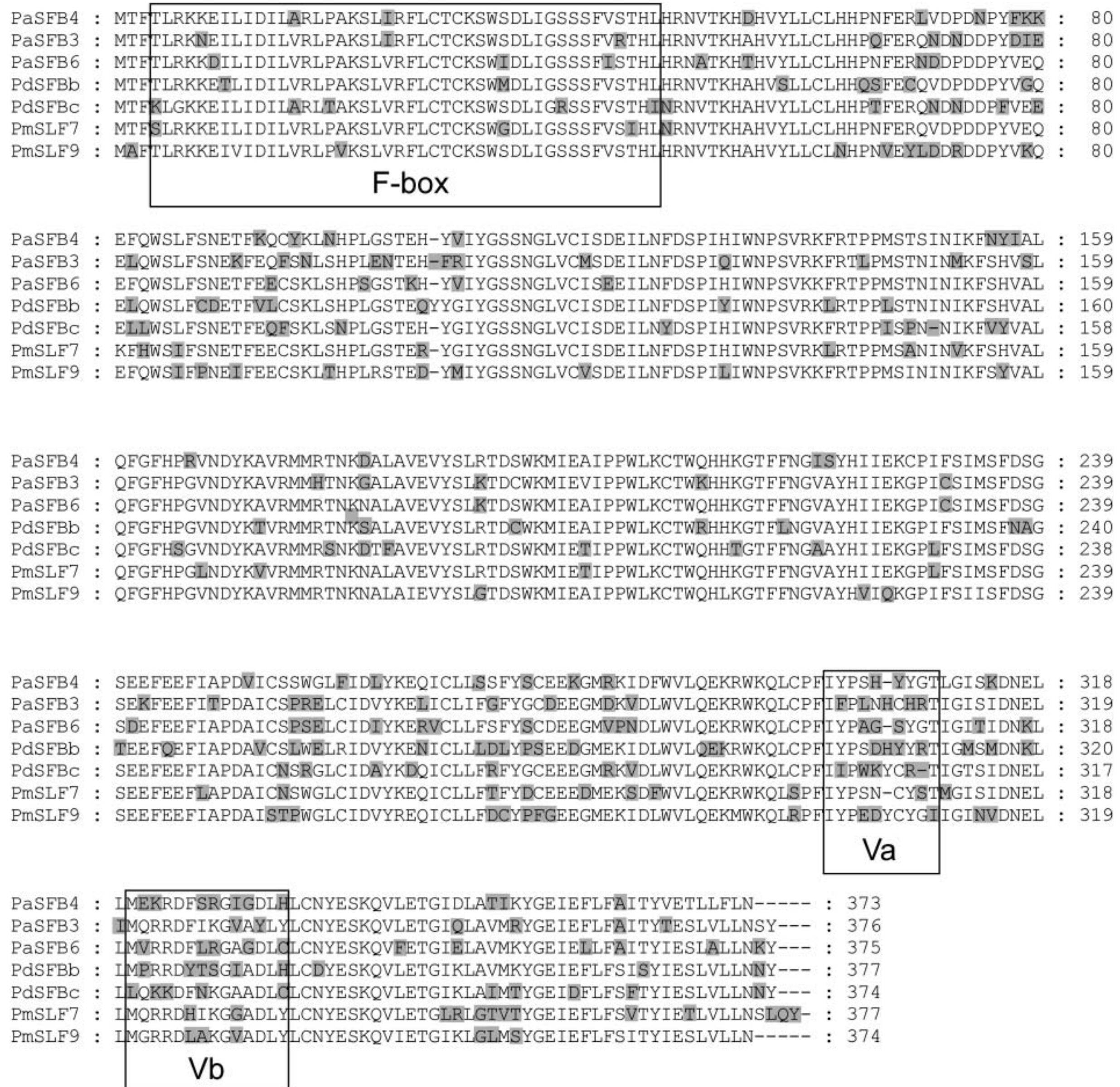

Figure 3. Alignment of the Predicted Amino Acid Sequence of the S_4-Haplotype–Specific *SFB* with Other Prunus *SFB*/*SLF* Sequences.

Seven *SFB* sequences of Prunus (Pa, *P. avium*; Pd, *P. dulcis*; Pm, *P. mume*) aligned using ClustalW. Residues highlighted in gray denote divergence from the observed consensus, and dashes represent gaps. The F-box region and two variable regions Va and Vb (as indicated in Ushijima et al., 2003) are boxed.

found in the $S_4{}'$-haplotype. When the coding region of the $S_4{}'$-*SFB* gene was cloned and the sequence aligned with the S_4-*SFB* sequence of the progenitor (Figure 10), a deletion of 4 bp was found toward the 3′ end of the $S_4{}'$ sequence (position 742 to 745) in the variable region A (as defined in Ushijima et al., 2003), which would result in a frame shift in translation. From that point onwards, the corresponding $S_4{}'$ protein contains 16 amino acids distinct from the S_4-SFB predicted protein sequence and then a premature stop codon would result in a truncated protein. Because the variable regions of the protein are altered significantly and the protein is truncated (317 versus 375 amino acids), the $S_4{}'$-SFB protein is unlikely to function properly.

DISCUSSION

A genetic analysis of two self-compatible cherry pollen-part mutants has confirmed that both selections have self-compatibility linked with one particular allele (i.e., S_3 in JI 2434 [denoted $S_3{}'$]

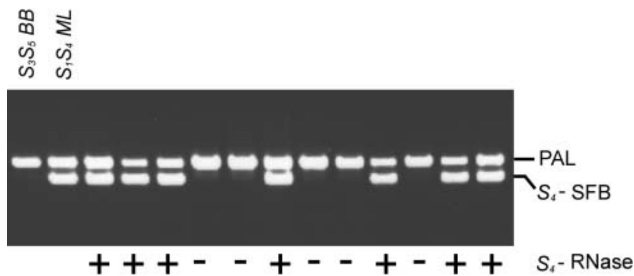

Figure 4. Segregation Analysis of the Putative S_4-Haplotype–Specific *SFB* Sequence to Test Cosegregation with the S_4-*RNase* Allele.

PCR amplification with specific primers for the putative S_4-specific *SFB* sequence is shown for genomic DNA of parents and a representative sample of seedlings of the cross Bradbourne Black (S_3S_5) × Merton Late (S_1S_4): BB, Bradbourne Black; ML, Merton Late; 12 seedlings. Presence (+) or absence (−) of the S_4-*RNase* allele is indicated underneath the lanes. The bands amplified by the internal control primers included in each PCR are indicated with PAL.

and S_4 in JI 2420 [denoted S_4']), confirming the conclusions of Bošković et al. (2000). A molecular characterization of *S* locus genes has shown that, in both mutant haplotypes, the *S-RNase* gene is intact, but each has a mutation affecting the haplotype-specific *SFB* gene. No evidence of a duplication was found in either mutant selection. In the S_3'-haplotype, a rearrangement has been found downstream of the *S-RNase* gene, with the breakpoint of the rearrangement ~3.5 kb from the *S-RNase* gene. In this rearrangement, the S_3-haplotype–specific *SFB* gene, which is located ~6.5 kb downstream of the S_3-*RNase* gene in the progenitor, is deleted. In the S_4'-haplotype, a 4-bp deletion in a variable region of the S_4-haplotype–specific *SFB* gene leads to a shift in the translation reading frame and premature termination of the protein.

Self-Compatibility Is Associated with Loss of Function of *SFB*

Prunus *SFB* genes have been reported as good candidates for the pollen component of SI. They are pollen-expressed genes with haplotype-specific polymorphism identified at the *S* locus in almond (*P. dulcis*) (Ushijima et al., 2003) and Japanese apricot (*P. mume*) (Entani et al., 2003). The amino acid identity between different alleles is comparable to *S-RNase* alleles (Entani et al., 2003; Ushijima et al., 2003), and like *S-RNases*, they show a *trans*-specific pattern of evolution (Ikeda et al., 2004). Our finding that two self-compatible pollen-part mutants of cherry are associated with a loss of function of the haplotype-specific *SFB* gene provides additional support that the *SFB* gene is the pollen-*S* gene in Prunus.

Loss of function of the S_3-*SFB* gene in the S_3'-haplotype is the result of a deletion of the gene (Figure 8). A variant of the S_4-SFB protein is still likely to be produced in the S_4' mutant, but the protein is truncated as the result of a premature stop codon (Figure 10). The C terminus of the S_4'-SFB protein will have 16 amino acids of novel sequence and 58 amino acids missing. These alterations are likely to have a significant effect on the protein structure and function. Even though the F-box domain at the N terminus may be unaffected, the variable domains at the C terminus, which are thought to be involved in specific interactions with proteins recruited for degradation (see later), would be changed or lost. The mutant protein is therefore unlikely to perform its normal specific function, and the mutation can be regarded as a loss-of-function mutation. During the preparation of this article, Ushijima et al. (2004) reported identical findings for the S_4'-haplotype. They speculate that the truncated S_4' protein, which lacks the two variable regions at the C terminus, may still function partially. Their conclusions are different from ours, as discussed later.

Previously, pollen-part mutations causing self-compatibility in plants with RNase-based SI have been found to be associated with duplications of *S* alleles (Brewbaker and Natarajan, 1960; Pandey, 1967; Golz et al., 1999, 2001). If *SFB* is indeed the pollen-*S* gene, the S_3' mutant characterized in this article would be an unambiguous example of loss of function of the pollen-*S* gene for an RNase-based SI system.

It is curious that the only two irradiation-induced pollen-part mutants identified in the Rosaceae, studied in this article, appear to be the result of loss-of-function mutations, whereas at least five pollen-part mutants of *P. inflata* (Brewbaker and Natarajan, 1960) and seven pollen-part mutants of *N. alata* (Golz et al., 1999, 2001), resulting from similar experiments in the Solanaceae, were attributable to duplications of an *S* allele, often on a centric fragment. Pandey (1965) also found that 34 pollen-part mutants of *N. alata* carried a centric fragment. This led Golz et al. (2001) to suggest that deletions of the pollen-*S* gene are incompatible on

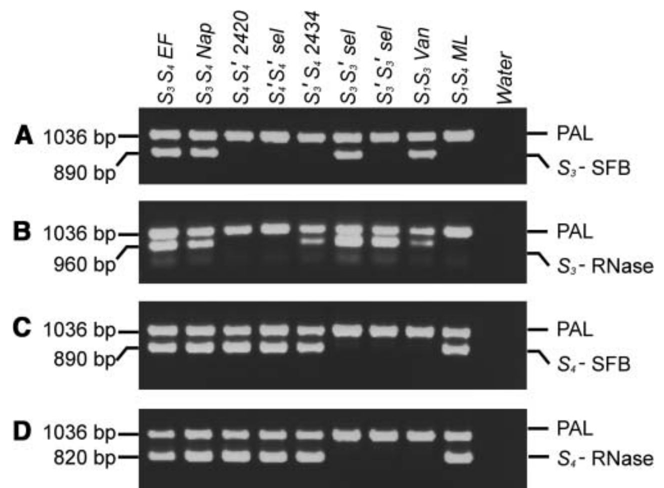

Figure 5. Genomic PCR Amplification of Parents and Pollen-Part Mutants with Specific Primers for the *SFB* and *S-RNase* Genes of the S_3- and S_4-Haplotypes.

The primers used are specific for S_3-haplotype–specific *SFB* **(A)**, S_3-*RNase* **(B)**, S_4-haplotype–specific *SFB* **(C)**, and S_4-*RNase* **(D)**. Samples are as follows: EF, Emperor Francis (S_3S_4); Nap, Napoleon (S_3S_4); 2420, JI 2420 (S_4S_4'); $S_4'S_4'$ selection; 2434, JI 2434 ($S_3'S_4$); S_3S_3' selection; $S_3'S_3'$ selection; Van (S_1S_3); ML, Merton Late (S_1S_4); water control. The bands amplified by the internal control primers that were included in each PCR are indicated with PAL.

Table 6. Presence (+) or Absence (−) of Genomic PCR Amplification in Parents and Pollen-Part Mutants with Specific Primers for the S_3- and S_4-*RNase* and -*SFB* Genes as Shown in Figure 5

Lane	Cultivar/Selection	*S*-Genotype	S_3-*SFB*	S_3-*RNase*	S_4-*SFB*	S_4-*RNase*
a	Emperor Francis	S_3S_4	+	+	+	+
b	Napoleon	S_3S_4	+	+	+	+
c	JI2420	S_4S_4'	−	−	+	+
d	A53	$S_4'S_4'$	−	−	+	+
e	JI 2434	$S_3'S_4$	−	+	+	+
f	9239-1	S_3S_3'	+	+	−	−
g	JI 2434 AH	$S_3'S_3'$	−	+	−	−
h	Van	S_1S_3	+	+	−	−
i	Merton Late	S_1S_4	−	−	+	+

any style with *S*-RNases. If their conclusion is valid, then finding a mutation/deletion of *SFB* in two pollen-part mutants would indicate that *SFB* is not the pollen-*S* gene.

However, it may be that certain *S*-bearing chromosomes in solanaceous species are particularly prone to breaking as a result of ionizing radiation. In these studies, it was often found that after irradiation of, for example, an S_3S_6 plant, most pollen-part mutants had a duplication of the S_3 allele, indicating that the mutation rate for some alleles is higher than for others. Also, centric fragments carrying the *S* locus may be frequent in solanaceous species because the *S* locus is known to be located at the centromere in Petunia (Entani et al., 1999), which may not be true for the *S* locus in Prunus. Lewis (1961) points out that the absence of the prime-type mutation in the studies of Brewbaker and Natarajan (1960) is not unexpected when taking into account the number of irradiated pollen grains involved and assuming that the mutation rate for the prime-type mutation in Petunia is the same as in his studies of Oenothera. However, it is currently unknown if Oenothera has RNase-based SI.

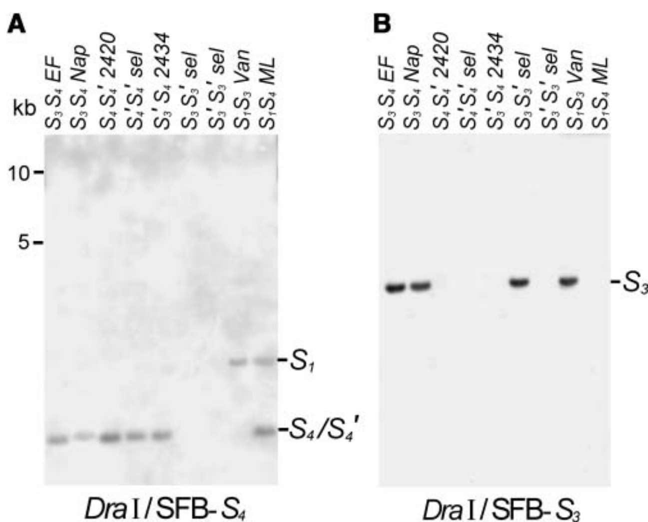

Figure 6. DNA Gel Blot Analysis of the S_3' and S_4' Mutants Using *SFB* Haplotype-Specific Probes.

Genomic DNA was digested with *Dra*I and probed with S_4-haplotype–specific *SFB* probe **(A)** and S_3-haplotype–specific *SFB* probe **(B)**. The stringent washing conditions resulted in an allele-specific hybridization signal with the S_3-*SFB* probe; the S_4-*SFB* probe also cross-hybridized weakly to the S_1-*SFB* sequence. Samples are as follows: EF, Emperor Francis (S_3S_4); Nap, Napoleon (S_3S_4); 2420, JI 2420 (S_4S_4'); $S_4'S_4'$ selection; 2434, JI 2434 ($S_3'S_4$); S_3S_3' selection; $S_3'S_3'$ selection; Van (S_1S_3); ML, Merton Late (S_1S_4).

It should be noted that a loss-of-function approach was not attempted in the transgenic experiments with *S* locus F-box genes reported in *P. inflata* (Sijacic et al., 2004) and Antirrhinum/*P. hybrida* (Qiao et al., 2004b), in which an extra copy of an *S* locus F-box gene was shown to cause breakdown of SI because of competitive interaction. This may have resulted from the assumption that these F-box proteins inactivate non-self *S*-RNases, in which case a loss of function should lead to pollen being incompatible on any style with *S*-RNases. The mode of action of the RNase-mediated SI reaction in the Rosaceae may not be fundamentally different from that in the Solanaceae or Scrophulariaceae because it has recently been suggested they have a common origin based on phylogenetic analyses of *S-RNase* sequences (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002). It will be interesting to find out whether knockout mutants of *S* locus F-box genes in the Solanaceae, which is more amenable to transformation than the Rosaceae, also become self-compatible. This would be another way to demonstrate that specific *S* locus F-box genes encode the pollen component of RNase-mediated SI in this family, in addition to the gain-of-function experiments reported so far.

Organization of the *S* Locus

The preliminary restriction map of the S_3-haplotype (Figure 8) shows that the *SFB* gene is located downstream of the *S-RNase* gene in opposite transcriptional direction, as was found in other Prunus *S* haplotypes, although distances may vary (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003). This organization is also found for the *AhSLF-S_2* and the *S_2-RNase* gene in Antirrhinum (Lai et al., 2002). By contrast, the two genes determining stigma and pollen specificity at the sporophytic *S* locus in Brassica are found in various relative orientations (Boyes et al., 1997; Cui et al., 1999; Watanabe et al., 2000). If additional *S* locus haplotypes continue this pattern, the convergent arrangement of the two genes at the *S* locus in families with RNase-based SI may be of functional significance.

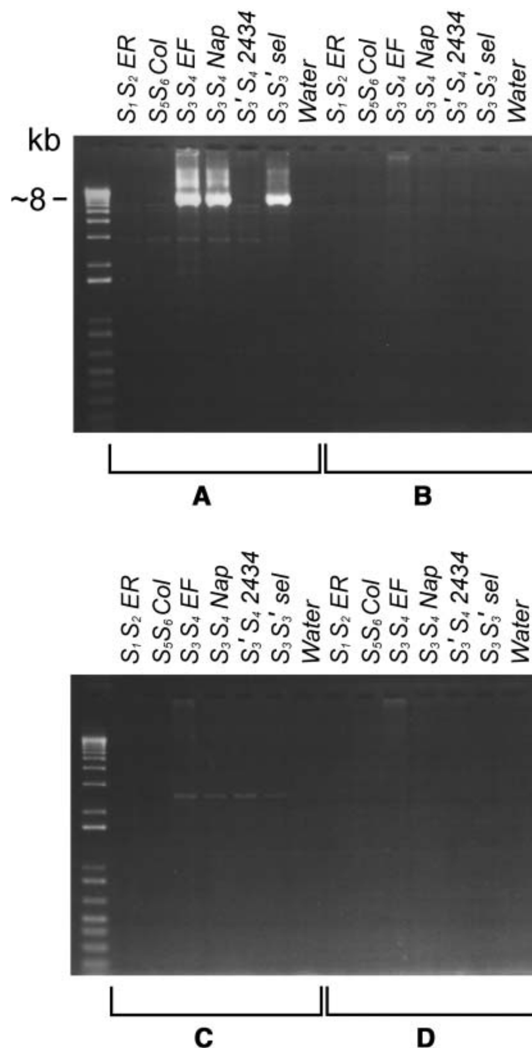

Figure 7. Intergenic PCR for the S_3-Haplotype Using S_3-*RNase* and S_3-*SFB* Specific Primers in Four Possible Combinations to Determine Distance between and Transcriptional Orientation of the Genes.

The primers used on genomic DNA are S_3-*RNase* forward with S_3-haplotype–specific *SFB* forward **(A)**, S_3-*RNase* forward with S_3-haplotype–specific *SFB* reverse **(B)**, S_3-*RNase* reverse with S_3-haplotype–specific *SFB* forward **(C)**, and S_3-*RNase* reverse with S_3-haplotype–specific *SFB* reverse **(D)**. Samples are as follows: ER, Early Rivers (S_1S_2); Col, Colney (S_5S_6); EF, Emperor Francis (S_3S_4); Nap, Napoleon (S_3S_4); 2434, JI 2434 ($S_3'S_4$); S_3S_3' selection; water control. Only combination **(A)** shows specific amplification for accessions with the S_3 allele.

In previous studies of pollen-part mutants in the Solanaceae, no breakpoint of rearrangements could be identified at a molecular level (Thompson et al., 1991; Golz et al., 1999, 2001). The size of the *P. avium* genome (2C = 686 Mbp) is only twice that of Arabidopsis (2C = 343 Mbp), whereas genomes of solanaceous species are much larger (e.g., *N. alata* 2C = 4753 Mbp; *P. hybrida* 2C = 3283 Mbp) (Bennett and Leitch Angiosperm DNA C-values database, release 4.0, January 2003, http://www.rbgkew.org.uk/cval/homepage.html). The smaller size of the Prunus genome may have increased the likelihood of detecting an irradiation-induced breakpoint. The relatively small genome size makes Prunus a suitable model for studies of the genomic organization of the *S* locus.

A wide range of mutations can be obtained from x-irradiation, such as deletions, translocations, inversions, and even point mutations. The rearrangement in the S_3'-haplotype appears to be relatively major, and preliminary analysis suggests a deletion. Sequencing of the *S-RNase/SFB* intergenic PCR product for the S_3-haplotype and comparing it to sequences downstream of the S_3-*RNase* in the S_3'-haplotype, obtained from an ~4.5-kb *Sst*I inverse PCR fragment, identified the breakpoint of the rearrangement ~3 kb from the *S-RNase* (data not shown). Primers based on the new flanking sequence in the S_3'-haplotype amplified a PCR product of unique size in samples with S_3 and S_3' and cosegregated with the S_3 allele in a set of 48 seedlings from the mapping progeny Napoleon (S_3S_4) × *P. nipponica* (S_aS_b) (Bošković and Tobutt, 1997) (data not shown), suggesting that the new flanking sequence originates from the S_3 locus. A deletion type rearrangement is therefore more likely than a translocation.

Other *S* locus F-box genes do not appear to be affected by the rearrangement. In particular, the presence of the *SLFL2* gene, which is located downstream of the *S-RNase* in other Prunus *S* haplotypes, including S_4 of cherry (Ushijima et al., 2004), flanked by the same restriction sites as in the S_3-haplotype, suggests that the S_3' rearrangement is not very extensive and may not affect other genes. The simplest interpretation is that the S_3'-haplotype has a deletion extending from ~3 kb downstream of *S*-RNase to a point in between *SFB* and *SLFL2*. However, this cannot be certain without further analysis of the entire S_3- and S_3'-haplotypes. No evidence of a rearrangement has been found by DNA gel blot analysis in sequences flanking the *S-RNase* and the *SFB* genes in the S_4'-haplotype and the 4-bp deletion in the coding region of the S_4'-*SFB* gene appears to be the only significant difference in the mutant S_4-haplotype.

Loss of Function of *SFB* and Models of RNase-Mediated SI

Although the mechanism of the RNase-based SI reaction is still largely unknown, the model currently favored is the RNase inhibitor model, variously proposed and developed by McClure et al. (1989), Thompson and Kirch (1992), Kao and McCubbin (1996), and Luu et al. (2000) (2001). Because pollen tubes appear to take up *S*-RNases indiscriminately (Luu et al., 2000), they must have a mechanism to inactivate the cytotoxic action of non-self *S*-RNases. In the original inhibitor model, the pollen component is assumed to be an RNase inhibitor inside the pollen tube, which is able to inhibit all *S*-RNases except the one of corresponding *S* genotype. In this model, pollen-*S* encodes a protein that has the two functions of inhibiting non-self *S*-RNases and conferring specificity. Luu et al. (2001) proposed a modification of the inhibitor model, in which the two functions of *S*-RNase inhibition and allele-specific recognition are carried by two separate proteins, a general RNase inhibitor, not necessarily encoded by the *S* locus, and pollen-*S*. Binding of pollen-*S* to its corresponding *S*-RNase would protect it from being inhibited by the general

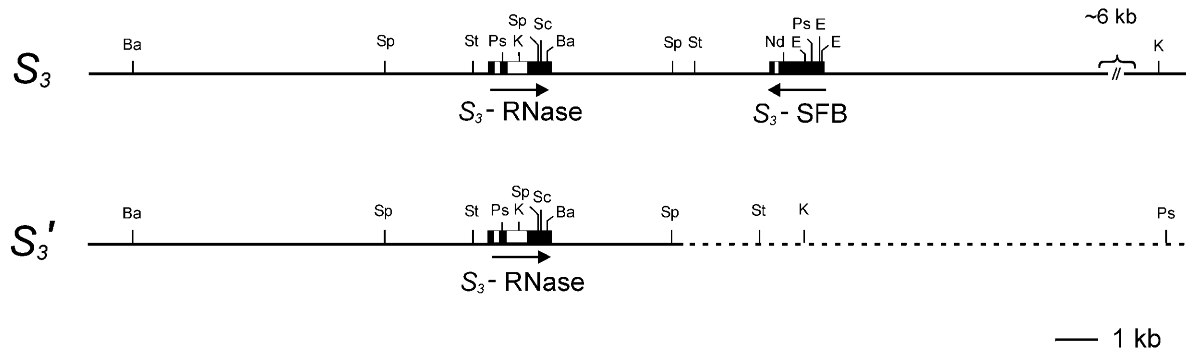

Figure 8. Preliminary Restriction Maps of the S_3- and S_3'-Haplotypes of Cherry of Napoleon (S_3S_4) and JI 2434 ($S_3'S_4$).

Restriction enzyme positions and distances were inferred from restriction fragment size estimates on DNA gel blots (Napoleon [S_3S_4] and JI 2434 [$S_3'S_4$]) (Table 5), except for the regions of known sequence. Genes are represented by black boxes, with white boxes for introns. Arrows underneath gene boxes indicate the direction of transcription as deduced from the intergenic PCR (Figure 7). The breakpoint of the rearrangement in the S_3'-haplotype was deduced to be ~3.5 kb downstream of the S_3-*RNase*; sequence downstream of the breakpoint (dotted line) is linked to S_3 in the progenitor. Restriction enzyme abbreviations are as follows: Ba, *Bam*HI; E, *Eco*RI; K, *Knp*I; Nd, *Nde*I; Ps, *Pst*I; Sc, *Sca*I; Sp, *Ssp*I; St, *Sst*I.

RNase inhibitor. The *S*-RNase therefore remains active and inhibits pollen tube growth. This version allows for deletions of pollen-*S*, in contrast with the original inhibitor model as pointed out by Golz et al. (2001), although to date there have been no unambiguous reports of pollen-*S* deletions in plants with RNase-based SI.

The recent findings of F-box proteins as candidates for the pollen-*S* component in three families with RNase-mediated SI have suggested that ubiquitin-mediated protein degradation by the 26S proteasome is involved in the mechanism to inactivate non-self *S*-RNases (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003; Sijacic et al., 2004). Biochemical studies in Antirrhinum have supported the role of ubiquitin-mediated protein degradation in compatible pollinations (Qiao et al., 2004a). They showed a nonallelic physical interaction of the *S* locus F-box protein AhSLF-S_2 with *S*-RNases. The SLF proteins were also found to interact with other proteins involved in the ubiquitin-mediated protein degradation pathway. In addition, proteasomal inhibitors blocked compatible pollinations but had little effect on incompatible pollinations. As pointed out by Zhou et al. (2003), it is difficult to establish whether the Antirrhinum *AhSLF* gene is the ortholog of *SFB* in Prunus, so whether studies in Antirrhinum apply to Prunus remains to be seen.

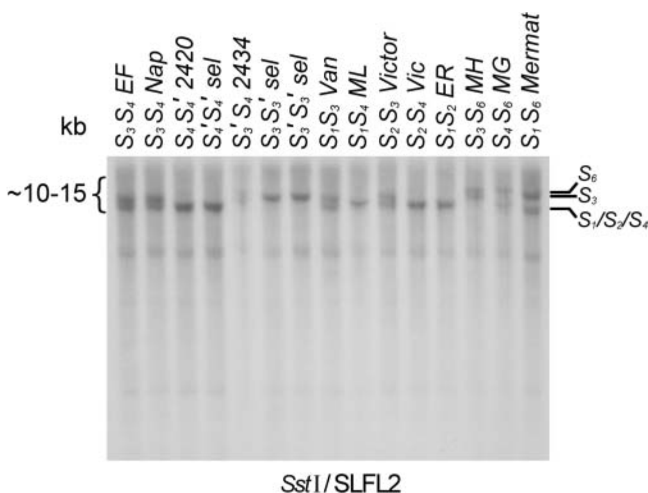

Figure 9. DNA Gel Blot Analysis of the S_3' and S_4' Mutants Using an *SLFL2*-Specific Probe.

Genomic DNA was digested with *Sst*I and probed with a cherry *SLFL2* probe. Samples are as follows: EF, Emperor Francis (S_3S_4); Nap, Napoleon (S_3S_4); 2420, JI 2420 (S_4S_4'); $S_4'S_4'$ selection; 2434, JI 2434 ($S_3'S_4$); S_3S_3' selection; $S_3'S_3'$ selection; Van (S_1S_3); ML, Merton Late (S_1S_4); Victor (S_2S_3); Vic (S_2S_4); ER, Early Rivers (S_1S_2); MH, Merton Heart (S_3S_6); MG, Merton Glory (S_4S_6); Mermat (S_1S_6).

In all three families, it has been proposed that the polymorphic *S*-locus F-box protein (SLF or SFB) is an inactivator of non-self *S*-RNases by recruiting non-self *S*-RNases as a substrate for destruction through the 26S proteasome pathway (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003, Sijacic et al., 2004). A different, allele-specific interaction (of unknown nature) of the F-box protein with self *S*-RNases would prevent these from being degraded. This is in accordance with the original inhibitor model in which the pollen-*S* component carries the two functions of *S*-RNase inhibition and allele-specific recognition. Ushijima et al. (2004), who recently reported that the cherry S_4' and *P. mume* S_f alleles encode truncated SFB proteins, attempt to reconcile their findings with this model by proposing that the truncated proteins lack the specific *S*-RNase interaction domain but retain a general *S*-RNase binding domain, leading to inactivation of all *S*-RNases, including the self *S*-RNase.

However, in the original inhibitor model, a loss of function of the haplotype-specific F-box gene should lead to universally incompatible pollen because pollen tubes would lack a mechanism to inhibit *S*-RNases. In this article, we have provided evidence of loss of function of *SFB* in at least one self-compatible mutant of cherry. Therefore, if *SFB* is the pollen-*S* gene in Prunus, it cannot have the role of inactivator of non-self *S*-RNases.

It seems more likely that SFB proteins provide specificity to the inactivation of *S*-RNases effected by a general inactivation mechanism present in pollen tubes. This would fit the

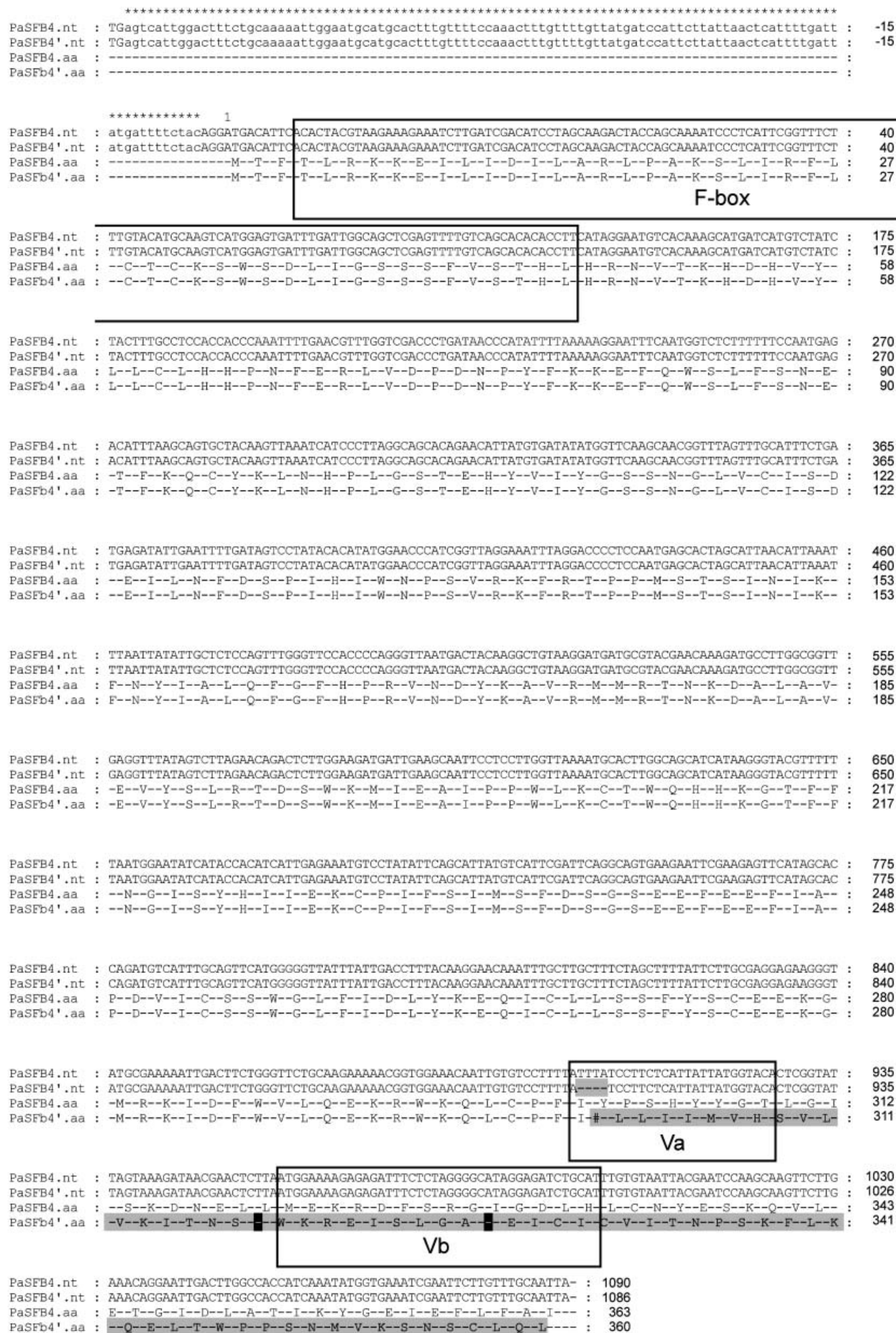

Figure 10. Alignment of Partial Genomic DNA Sequences and Deduced Amino Acid Sequences of the S_4- and S_4'-Haplotype–Specific *SFB* Genes.

Nucleotide (nt) and predicted amino acid (aa) sequences representing *P. avium SFB* sequences for S_4 and S_4'. Putative intron sequence within the 5′ untranslated region is presented in lower case and indicated by asterisks. Twelve amino acids are missing at the 3′ end of the S_4 sequence. Residues highlighted in gray denote divergence from the original S_4-*SFB* sequence. The F-box region and the two variable regions Va and Vb as identified by Ushijima et al. (2003) are boxed. A 4-bp deletion in the S_4'-*SFB* sequence at position 742 to 745 in the first variable region leads to the deletion of a single Tyr residue (#) and a subsequent frame shift in translation. Premature stop codons present in the S_4'-*SFB* sequence are highlighted in black.

two-component inhibitor model (Luu et al., 2000). Deletions of the *SFB* gene would then result in pollen tubes able to inactivate all *S*-RNases, leading to self-compatibility. In that case, SFB proteins should only prevent self *S*-RNases from being degraded (not recruit non-self *S*-RNases for degradation), and so it is difficult to understand the function of their F-box domain. The F-box domain suggests that SFB may act as part of an E3 ubiquitin ligase complex in targeted protein degradation via the 26S proteasome. However, E3 ubiquitin ligase activity has not been demonstrated for SFB, and it may function in an unexpected way. As discussed, Qiao et al. (2004) provide evidence that ubiquitin/26S proteasome activity is essential in compatible but not in incompatible interactions in Antirrhinum. This suggests that a general inhibitor may use the ubiquitin/26S proteasome pathway of protein degradation for the inactivation of non-self *S*-RNases, but that in incompatible interactions, in which self *S*-RNases are protected from being degraded, the ubiquitin/26S proteasome pathway is not involved.

Sims and Ordanic (2001) first suggested that ubiquitination and protein degradation may play a role in the SI reaction in *P. hybrida*. In a yeast two-hybrid assay, they identified a pollen-expressed protein (PhSBP1) with a RING-finger domain that binds to *S*-RNases in a non-allele–specific manner. Many proteins with a RING-finger domain participate in E3 ubiquitin ligase complexes, like F-box proteins, and they suggest that PhSBP1 is a candidate for the general inhibitor of *S*-RNases, the existence of which was proposed in the two-component inhibitor model of Luu et al. (2000). It may be that a RING-finger domain protein with E3 ubiquitin ligase activity exists also in Prunus as a general inactivator of *S*-RNases.

It is clear that other components are involved in the complex interactions of the SI reaction, such as perhaps an *S*-RNase binding RING-finger domain protein homologous to PhSBP1 as just discussed or other pollen-expressed F-box proteins that are known to be encoded by the *S* locus (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003; Sijacic et al., 2004), although Qiao et al. (2004) report that other F-box proteins identified in Antirrhinum do not interact physically with *S*-RNases. Further biochemical investigations using the mutants identified in this article and their progenitors are needed to shed light on the mechanism of *S*-RNase inactivation and the precise role of SFB in protecting self *S*-RNases from inactivation.

Self-Compatibility and Cherry Breeding

JI 2420, the S_4' mutant, has been used successfully in breeding programs combining self-compatibility with high fruit quality. One of its seedlings, Stella, was the first self-compatible cherry cultivar released, and nearly all other self-compatible cherry cultivars that are now available derive from Stella. JI 2434 has not been used extensively in breeding, but it may be in the parentage of Alex. Although the recorded parentage of Alex indicates a distinct mutant selection, JI 2538, the S_3' allele detected (Sonneveld et al., 2003) displays the same *Eco*RI shift on DNA gel blots with the *S-RNase* probe as the S_3' allele of JI 2434 (data not shown).

Self-compatibility is an important agronomic character in sweet cherry, and a PCR-based test can now be developed for the mutant *S* haplotypes, so seedlings can be selected for self-compatibility soon after germination. Previously, this had to be done by detecting the *S-RNases* in these haplotypes, which do not differ from the normal S_3 and S_4 alleles, and self-compatibility had to be deduced from the parentage of the seedlings (Bošković et al., 1997, 2000; Sonneveld et al., 2001, 2003).

METHODS

Plant Material

Two cherry (*Prunus avium*) pollen-part mutant selections raised at the John Innes Institute from the nominally incompatible cross of the cultivars Emperor Francis (S_3S_4) × Napoleon (S_3S_4, x-ray pollen) were used in this investigation, JI 2420 and JI 2434, with mutations affecting the S_4 and the S_3 alleles, respectively (Lewis, 1949; Matthews and Lapins, 1967; Matthews, 1970). The particular clones used were JI 2420 with genotype S_4S_4' and JI 2434 EM with genotype $S_3'S_4$ (Bošković et al., 2000). The JI 2434 AH clone, which was subsequently found to be a possible self of JI 2434 EM with the genotype $S_3'S_3'$ (data not shown), was used as an example of an $S_3'S_3'$ homozygote.

To confirm that the pollen-part mutation in JI 2420 is linked with S_4, the progeny of Erika (S_1S_3) × Lapins (S_1S_4') [Van × Stella (JI 2420 × Lambert)] was scored in 1999 using the stylar ribonuclease assay of Bošković and Tobutt (1996). To confirm that self-compatibility in JI 2434 is linked with S_3, the backcross Van (S_1S_3) × 9239-3 (S_1S_3') [Van × JI 2434 ($S_3'S_4$)] was made in 2000 and the progeny genotyped by PCR using consensus primers for the first intron of cherry *S-RNases* (Sonneveld et al., 2003).

The S_4- and S_4'-*SFB* genes were cloned from the cultivars Inge (S_4S_9) [and later also Napoleon (S_3S_4)] and Sonata (S_3S_4'), respectively. Sonata, Lapins (S_3S_4') × [Van × Stella (S_3S_4')] is derived from JI 2420 via Lapins and Stella. Cosegregation of the putative S_4-*SFB* sequence with S_4 was tested in 44 seedlings of family 9007, Bradbourne Black (S_3S_5) × Merton Late (S_1S_4).

The following samples were included in DNA gel blot and *S* allele-specific PCR analyses: JI 2420 (S_4S_4'), JI 2434 ($S_3'S_4$), Emperor Francis (S_3S_4), and Napoleon (S_3S_4), along with standards for S_3 and S_4, Van (S_1S_3) and Merton Late (S_1S_4), respectively. In addition, for some of the analyses, four selections from family 9239 [Van (S_1S_3) × JI 2434 ($S_3'S_4$)] with genotypes S_1S_3', S_3S_3', S_1S_4, and S_3S_4, or only the S_3S_3' selection, were used. A selection homozygous for S_4', A53 [Stella (S_3S_4') × self] (Bošković et al., 1998), and one homozygous for S_3' (JI 2434 AH) were also included.

All plant material was grown at East Malling Research (UK), except for the progeny of Erika × Lapins, which was supplied by BAZ (Ahrensburg, Germany).

DNA Extraction

Cherry genomic DNA for DNA gel blotting was extracted from winter buds essentially as described by Dellaporta et al. (1983). DNA for genomic PCR and cloning was extracted from two dormant buds using a scaled down cetyl-trimethyl-ammonium bromide extraction method (Doyle and Doyle, 1987) with the addition of 1% (v/v) β-mercaptoethanol and 2% (w/v) polyvinyl pyrollidone (PVP 40) to the extraction buffer.

DNA Gel Blotting

Four micrograms of genomic DNA was digested overnight with the enzymes listed in Table 5 (*S-RNase* blots), *Dra*I (*SFB* blot), or *Dra*I and *Sst*I (SLFL blots). The fragments were separated on 0.8% agarose gels for

~24 h at 25 V and blotted onto a positively charged nylon membrane according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN).

DIG Labeling of Probe

To detect S_1-, S_3-, and S_4-*RNases*, an S_1-*RNase* cDNA product from the C2 to the C5 region (420 bp) was used as a probe. The region covered by the S_1 probe has 82.4% sequence similarity to both S_3- and S_4-*RNase*. The probe was labeled with Digoxigenin-11-dUTP (DIG-dUTP; Roche Molecular Biochemicals) by PCR with the consensus primer pair PaConsII-F and PaConsII-R (Sonneveld et al., 2003). The PCR reaction mix contained the following: 1× PCR buffer, 2 mM magnesium chloride, 50 μM dATPs, dCTPs, and dGTPs, 39.6 μM dTTPs, 10.5 μM DIG-dUTPs, 0.2 μM of each of the primers, 1.25 units/50 μL reaction *Taq* DNA polymerase (Qiagen, Crawley, UK), and ~20 to 100 pg plasmid DNA containing S_1-*RNase* cDNA (Sonneveld et al., 2001). Cycling conditions were as follows: initial denaturing of 2 min at 95°C, 10 cycles of 10 s at 95°C, 30 s at 57°C, and 2 min at 72°C, 20 cycles of 10 s at 95°C, 30 s at 57°C, and 2 min plus 5 s/cycle at 72°C, followed by 7 min at 72°C.

S_3- and S_4-*SFB* genomic PCR products were used as the probes to detect the S_3- and the S_4-*SFB*, respectively. The *SFB*-specific primers listed below under "Genomic PCR with *S-RNase* and *SFB*-Specific Primers" were used to amplify the regions from genomic DNA. The labeling reaction was the same as for the *S-RNase* probe, except for the annealing temperature of the PCR, which was 55°C.

SLFL2-specific primers designed from the *P. mume* S_1 and S_7 *SLFL2* sequences (AB092625 and AB092626), SLFL2-forward (5′-TGGCAACSTTGAGCAAATTTTCTG-3′) and SLFL2-reverse (5′-CTATCTCATCGTCGTCGTCTTC-3′), were used for amplification of a probe from genomic DNA of Napoleon.

Hybridization and Chemiluminescent Detection of DIG-Labeled Probe

Membranes were hybridized according to the DIG system User's Guide for Filter Hybridizations (Roche Molecular Biochemicals), with 3 μL probe per mL DIG Easy Hyb hybridization buffer. Low stringency post-hybridization washes of membranes that were hybridized with the *S-RNase* probe allowed the detection of cross-hybridization of the S_1-*RNase* probe to other *S-RNase* alleles: two washes of 5 min each with 2× SSC, 0.1% SDS at room temperature, followed by two washes of 15 min each with 0.5× SSC, and 0.1% SDS at 68°C. For membranes hybridized with the *SFB* probes, more stringent washes were used to achieve an allele-specific hybridization signal: two washes of 5 min each with 2× SSC, 0.1% SDS at room temperature, followed by two washes of 15 min each with 0.1× SSC, and 0.1% SDS at 68°C. For the chemiluminescent detection, the substrate CDP-Star (Sigma-Aldrich, Poole, UK) was used.

Cloning S_4- and S_4'-Haplotype-Specific *SFB* Genes

Sequence homology analysis was performed using four *SFB* genes identified in *P. dulcis* (AB092966, AB092967, AB079776, and AB081648; Ushijima et al., 2003) and three homologous *SLF* genes identified in *P. mume* (AB092621, AB092622, and AB092645; Entani et al., 2003) using the DNAstar Megalign (Madison, WI) software. Degenerate primers FBOX5′A (5′-TTKSCHATTRYCAACCKCAAAAG-3′) and FBOX3′A (5′-WATTGAGWAARRSYAAASTTTCTA-3′) were designed to anneal within conserved regions identified upstream of a putative intron within the 5′ untranslated region and towards the 3′ end of the coding sequence, respectively.

Amplification products representing genomic *SFB* clones were generated using proofreading KOD DNA polymerase (Invitrogen, Paisley, UK) in PCR using 100 ng of genomic DNA as template in a 30 μL reaction mix (1× KOD buffer, 0.2 mM dNTP, 1 mM magnesium sulfate, 0.5 μM forward and reverse primer, and 1 unit of KOD polymerase). PCR cycling conditions were 95°C for 2 min followed by 10 cycles of 94°C for 30 s, 60°C for 60 s with a reduction in temperature of 1°C per cycle, 68°C for 90 s then, 25 cycles of 94°C for 30 s, 50°C for 60 s, 68°C for 90 s, and a final cycle of 68°C for 10 min.

Amplification products were size fractionated by electrophoresis and DNA extracted from agarose using the QIAEX II kit (Qiagen). Amplification products generated from each cultivar were subsequently cloned into the vector pCR4-TOPO (Invitrogen) and transformed into TOP10 chemically competent cells (Invitrogen). Plasmid DNA was then prepared using a mini-spin kit (Qiagen) and inserts sequenced using M13 forward and reverse primers and internal primer, FBOX360 (5′-AGAATTTCAATGGTCTCTTTTTTTCC-3′). DNA from four separate colonies containing S_4'-*SFB* was sequenced to substantiate fully the data collected.

Sequences for *SFB* sequences for S_4 and S_4' have been submitted to the EMBL database, and accession numbers are AY649872 and AY649873, respectively. The S_4 sequence was initially cloned from the cultivar Inge (S_4S_9), but we later obtained an identical sequence from Napoleon (data not shown). Our S_4 sequences are identical to the S_4-*SFB* sequence now in the database (Ikeda et al., 2004).

The following sequences were used for the alignment of Figure 3: EMBL/GenBank accession numbers PaSFB4 (AY649872), PaSFB3 (AB096857), PaSFB6 (AB096858), PdSFBb (AB092967), PdSFBc (AB079776), PmSLF7 (AB092622), and PmSFB9 (AB092645).

Primers specific for the putative S_4-*SFB* sequence (see next section) were used in genomic PCR to test cosegregation with the S_4 allele in the family 9007 Bradbourne Black (S_3S_5) × Merton Late (S_1S_4), as described previously for the S_4-*RNase* specific primers (Sonneveld et al., 2001).

Genomic PCR with *S-RNase* and *SFB*-Specific Primers

PCR analysis of various selections and cultivars with S_3-*RNase* and S_4-*RNase* specific primers and an internal control was performed as described by Sonneveld et al. (2001) (2003); annealing temperatures of 60°C (S_4-*RNase*) and 63°C (S_3-*RNase*) were used. Specific primers for the S_3- and the S_4-*SFB* sequences (S_3-*SFB*, EMBL database accession number AB096857; S_4-*SFB*, AY649872) were designed: PaSFB3-F (S_3-*SFB* forward) 5′-CCACAATTTGAACGTCAGAAC-3′; PaSFB3-R (S_3-*SFB* reverse), 5′-GATTTCGCCATATCTCATGAC-3′; PaSFB4-F (S_4-*SFB* forward), 5′-TTGAACGTTTGGTCGACC-3′; PaSFB4-R (S_4-*SFB* reverse), 5′-TACACAAATGCAGATCTCCTATG-3′. The PAL internal control primers described by Sonneveld et al. (2003) were also included in the PCR with *SFB* primers. PCR conditions for the *SFB* primers were as described for the *S-RNase* allele-specific primers, with an annealing temperature of 54°C.

Intergenic PCR

PCR conditions were based on the Qiagen protocol for long PCR products. Approximately 100 ng of genomic DNA was used in a reaction mix containing the following: 1× PCR buffer (Qiagen), a final concentration of 2 mM magnesium chloride, 0.2 mM dNTPs, 0.2 μM of each of the primers, and 1.25 units/25 μL reaction *Taq* DNA polymerase (Qiagen). Cycling conditions were as follows: initial denaturing of 2 min at 94°C, 10 cycles of 10 s at 94°C, 1 min 30 s at 55°C, and 6 min at 68°C, 25 cycles of 10 s at 94°C, 1 min 30 s at 55°C, and 6 min plus 10 s/cycle at 68°C.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY649872 and AY649873.

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