Molecular and Genetic Analyses of Four Nonfunctional *S* Haplotype Variants Derived from a Common Ancestral *S* Haplotype Identified in Sour Cherry (*Prunus cerasus* L.)

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ABSTRACT

Tetraploid sour cherry (*Prunus cerasus*) has an S-RNase-based gametophytic self-incompatibility (GSI) system; however, individuals can be either self-incompatible (SI) or self-compatible (SC). Unlike the situation in the Solanaceae, where self-compatibility accompanying polyploidization is often due to the compatibility of heteroallelic pollen, the genotype-dependent loss of SI in sour cherry is due to the compatibility of pollen containing two nonfunctional *S* haplotypes. Sour cherry individuals with the *S*₄*S*₆*S*_{36a}*S*_{36b} genotype are predicted to be SC, as only pollen containing both nonfunctional *S*_{36a} and *S*_{36b} haplotypes would be SC. However, we previously found that individuals of this genotype were SI. Here we describe four nonfunctional *S*₃₆ variants. Our molecular analyses identified a mutation that would confer loss of stylar *S* function for one of the variants, and two alterations that might cause loss of pollen *S* function for all four variants. Genetic crosses showed that individuals possessing two nonfunctional *S*₃₆ haplotypes and two functional *S* haplotypes have reduced self-fertilization due to a very low frequency of transmission of the one pollen type that would be SC. Our finding that the underlying mechanism limiting successful transmission of genetically compatible gametes does not involve GSI is consistent with our previous genetic model for *Prunus* in which heteroallelic pollen is incompatible. This provides a unique case in which breakdown of SI does not occur despite the potential to generate SC pollen genotypes.

GAMETOPHYTIC self-incompatibility (GSI) is a widespread mechanism in flowering plants that prevents self-fertilization and promotes out-crossing (DE NETTANCOURT 2001). In GSI plants, pollen tube growth is arrested if there is a match between the genes at the *S*-locus that control pollen and stylar specificity. The gene controlling stylar specificity in the Solanaceae, Rosaceae, and Plantaginaceae is known to encode a ribonuclease (S-RNase) (for a review see McCLURE 2009), while the gene controlling pollen specificity encodes an F-box protein [*S* haplotype-specific F-box

protein (SFB) or *S*-locus F-box protein (SLF)] (LAI *et al.* 2002; ENTANI *et al.* 2003; USHIJIMA *et al.* 2003; SIJACIC *et al.* 2004). As these two specificity genes are tightly linked and recombination between these two genes has never been observed (IKEDA *et al.* 2005), these two *S*-locus specificity genes are collectively termed the *S* haplotype.

Characterization of the *S* haplotype is most advanced in *Prunus* (Rosaceae) due to the small physical size of the *S* haplotype region and the close proximity of the stylar *S* (*S*-RNase) and pollen *S* (*SFB*) genes (ENTANI *et al.* 2003; USHIJIMA *et al.* 2003; YAMANE *et al.* 2003b; IKEDA *et al.* 2005). Within *Prunus*, sweet cherry (*Prunus avium*) and sour cherry (*P. cerasus*) represent a model diploid-tetraploid series that has been used to investigate the effects of polyploidy on GSI. Tetraploid sour cherry is considered to have arisen through hybridization between sweet cherry and tetraploid ground cherry (*P. fruticosa*) (OLDEN and NYBOM 1968). Like sweet cherry, sour cherry exhibits an S-RNase-based GSI system (YAMANE *et al.* 2001; HAUCK *et al.* 2002; TOBUTT *et al.* 2004) and interspecific crossing studies have demonstrated that sour cherry shares eight sweet cherry *S* haplotypes: *S*₁, *S*₄, *S*₆, *S*₉, *S*₁₂, *S*₁₃, *S*₁₄, and *S*₁₆ (BOŠKOVIĆ *et al.* 2006; HAUCK *et al.* 2006a,b; TSUKAMOTO *et al.* 2006, 2008). However, in contrast to sweet cherry, natural sour

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Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. EU042127 (*S*_{36a} RNase), EU042128 (*S*_{36b} RNase), EU042129 (*S*_{36b2} RNase), EU042130 (*S*_{36b3} RNase), EU042131 (*SFB*_{36a}), EU042132 (*SFB*_{36b} obtained from *S*_{36b} haplotype), EU042133 (*SFB*_{36b} obtained from *S*_{36b2} haplotype), and EU042134 (*SFB*_{36b} obtained from *S*_{36b3} haplotype).

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cherry selections include both self-incompatible (SI) and self-compatible (SC) types. A genetic model demonstrating that the genotype-dependent loss of SI in sour cherry is due to the accumulation of a minimum of two nonfunctional *S* haplotypes within a single individual was developed and validated (HAUCK *et al.* 2006b). These nonfunctional *S* haplotypes were characterized as either pollen-part mutants or stylar-part mutants, depending on whether the pollen *S* or stylar *S* specificity was disrupted. In *Prunus*, pollen-part and stylar-part mutants are denoted by a prime symbol “'” or a subscripted “*m*,” respectively, following the *S* haplotype number (TSUKAMOTO *et al.* 2006). Molecular characterizations of five of the nonfunctional *S* haplotypes from sour cherry characterized to date support the genetic results because mutations were identified that affected the *S*-RNase and/or *SFB*. These changes in coding or regulatory regions included mutations within the *S*-RNase and/or *SFB* causing premature stop codons, transposable element insertions within *SFB* and upstream of the *S*-RNase, and a 23-bp deletion in a conserved region of the *S*-RNase (YAMANE *et al.* 2003a; HAUCK *et al.* 2006a; TSUKAMOTO *et al.* 2006).

According to the genetic model, termed the “one-allele-match model,” sour cherry pollen is rejected if one or both of the functional *S* haplotypes in the 2x pollen grain match an *S* haplotype in the style (HAUCK *et al.* 2006b). Therefore, only pollen containing two nonfunctional *S* haplotypes would be SC; thus, a sour cherry genotype is SC if it has a minimum of two nonfunctional *S* haplotypes. We previously tested the one-allele-match model using 92 sour cherry selections from four progeny populations (HAUCK *et al.* 2006b). For all the progeny except three, their *S* genotype correctly predicted whether they were SI or SC. The three progeny individuals that were the exception all had the same genotype: *S*₄*S*₆*S*_a*S*_d. These individuals were predicted to be SC as the *S*_a and *S*_d haplotypes were shown to be nonfunctional in genetic studies and therefore *S*_a*S*_d pollen should be SC. However, these progeny were classified as SI on the basis of observations of self-pollen tube growth in the styles. The *S*_a and *S*_d haplotypes were originally distinguished on the basis of different RFLP fragment sizes using an *S*-RNase probe; the *Hind*III fragment sizes for *S*_a and *S*_d differed by ~200 bp, 6.4-kb and 6.2-kb, respectively (YAMANE *et al.* 2001; HAUCK *et al.* 2002). However, partial *S*-RNase and *SFB* sequences from the *S*_a and *S*_d haplotypes were identical (N. R. HAUCK and A. F. IEZZONI, unpublished results), suggesting that *S*_a and *S*_d represented different mutations of the same *S* haplotype. Therefore, we hypothesized that the SI phenotype of the *S*₄*S*₆*S*_a*S*_d individuals resulted from complementary pistil *S* and pollen *S* mutations in the nonfunctional *S*_a and *S*_d haplotypes, thus behaving genetically as one functional *S* haplotype.

We previously reported that heteroallelic sour cherry pollen containing two different functional pollen *S*

haplotypes is incompatible (HAUCK *et al.* 2006b). This finding is counter to the well-documented phenomenon in the Solanaceae where SC accompanying polyploidization is frequently due to the SC of heteroallelic pollen (LEWIS 1943; GOLZ *et al.* 1999, 2001; TSUKAMOTO *et al.* 2005; XUE *et al.* 2009). Therefore, models explaining the molecular basis of self-recognition in *Prunus* and the Solanaceae must be consistent with these differing genetic expectations. Recently, HUANG *et al.* (2008) reported competitive interaction in a SC selection of tetraploid *P. pseudocerasus*, raising the possibility that the SC mechanism between these two tetraploid *Prunus* species could be different. However, although the data in HUANG *et al.* (2008) are consistent with heteroallelic pollen being SC, homoallelic pollen (*e.g.*, *S*₁*S*₁, *S*₅*S*₅, or *S*₇*S*₇) was not shown to be successful in compatible crosses and unsuccessful in incompatible ones. Therefore, it is possible that the SC in *P. pseudocerasus* could be caused by mutations in other genes critical for the SI reaction. Because of the importance of these differing genetic expectations for understanding *S*-RNase-based GSI, we sought to investigate our previously identified exceptions to the one-allele-match model. Specifically, our objective was to test our prior hypothesis that the nonfunctional *S*_a and *S*_d haplotypes interact in a complementary manner and therefore behave together genetically as a single functional *S* haplotype. In this work, the *S*_a and *S*_d haplotypes were renamed *S*_{36a} and *S*_{36b}, respectively, following the order of previously published *S* haplotypes (TSUKAMOTO *et al.* 2008; VAUGHAN *et al.* 2008) for reasons explained in the results.

MATERIALS AND METHODS

Plant material and DNA extraction: Twenty-one sour cherry cultivars and 6 SI sweet cherry cultivars were used in this study (Table 1). These cultivars were grown at the Michigan State University Clarksville Horticultural Experimental Station (Clarksville, MI). Young leaves were collected in the spring, frozen in liquid nitrogen, lyophilized, and stored at -20° until use. Genomic DNA was isolated from lyophilized leaves according to the method of IKEDA *et al.* (2004b). Extracted leaf DNA was treated with RNase A (Roche, Mannheim, Germany) and was used for PCR and fosmid library construction.

Southern blotting: Total DNA was isolated from young leaves by the cetyltrimethylammonium bromide (CTAB) method described by STOCKINGER *et al.* (1996). Genomic DNA blot analysis was performed as described by HAUCK *et al.* (2001). Probe cDNA was prepared from the PCR-amplified fragment of the *S*₆-RNase cDNA from sweet cherry and radiolabeled with [³²P]dCTP as described by HAUCK *et al.* (2001).

Construction and screening of genomic libraries: Fosmid libraries were constructed using the Copy Control Fosmid library production kit (Epicentre Technologies, Madison, WI). Fosmid libraries from Rheinische Schattenmorelle, Újfehértói fürtös, Meteor, Erdi Naggyumolcsu, Cigány 59, and MSU III 18 (12) were constructed to clone the *S*-RNase and *SFB* alleles for the *S*_{36a}, *S*_{36b}, *S*_{36b2}, and *S*_{36b3} haplotypes (Table 1). These

TABLE 1
Pollination phenotypes and S genotypes for 21 sour cherry and 6 sweet cherry cultivars used in this study

Cultivar	Phenotype ^a	S genotype ^{b,c}	Reference for S genotype
Sour cherry			
Cigány 59	SC	$S_{6m2}S_9S_{26}S_{36b2}$	YAMANE <i>et al.</i> (2001), HAUCK <i>et al.</i> (2006b), TSUKAMOTO <i>et al.</i> (2006)
Crisana	SI	$S_1S_4S_{35}S_{36b}$	YAMANE <i>et al.</i> (2001), TSUKAMOTO <i>et al.</i> (2008)
Englaise Timpurii	SC	$S_{13}'S_{14}S_{36a}S_{36b}$	TSUKAMOTO <i>et al.</i> (2008)
Erdi Botermo	SC	$S_4S_{6m}S_{35}S_{36a}$	HAUCK <i>et al.</i> (2006b), TSUKAMOTO <i>et al.</i> (2008)
Erdi Jubileum	SC	$S_1S_6S_{13}'S_{36b}$	TSUKAMOTO <i>et al.</i> (2008)
Erdi Nagygyumolcsu	SI	$S_1S_{12}S_{13}S_{36b}$	YAMANE <i>et al.</i> (2001)
Meteor	SC	$S_{13m}S_{33}S_{36a}S_{36b}$	TSUKAMOTO <i>et al.</i> (2008)
Montmorency	SC	$S_6S_{13m}S_{35}S_{36a}$	YAMANE <i>et al.</i> (2001), HAUCK <i>et al.</i> (2006b), TSUKAMOTO <i>et al.</i> (2006, 2008)
Pandy 38	SI	$S_1S_4S_{35}S_{36b}$	YAMANE <i>et al.</i> (2001), HAUCK <i>et al.</i> (2006b), TSUKAMOTO <i>et al.</i> (2008)
Pandy 114	SI	$S_1S_4S_{35}S_{36b}$	YAMANE <i>et al.</i> (2001), HAUCK <i>et al.</i> (2006b), TSUKAMOTO <i>et al.</i> (2008)
Rheinische Schattenmorelle	SC	$S_6S_{13}'S_{26}S_{36a}$	YAMANE <i>et al.</i> (2001), HAUCK <i>et al.</i> (2006b), TSUKAMOTO <i>et al.</i> (2006)
Surefire	SC	$S_4S_{13}'S_{35}S_{36a}$	YAMANE <i>et al.</i> (2001), HAUCK <i>et al.</i> (2006b), TSUKAMOTO <i>et al.</i> (2008)
Tamaris	SC	$S_{13}'S_{16}S_{34}S_{36b2}$	TSUKAMOTO <i>et al.</i> (2008)
Tarina	SI	$S_1S_4S_{36a}S_{36b}$	TSUKAMOTO <i>et al.</i> (2008)
Tschernokorka	SI	$S_9S_{13}S_{35}S_{36b2}$	YAMANE <i>et al.</i> (2001), TSUKAMOTO <i>et al.</i> (2008)
Újfehértói fűrtös	SC	$S_1'S_4S_{35}S_{36b}$	YAMANE <i>et al.</i> (2001), HAUCK <i>et al.</i> (2006a,b), TSUKAMOTO <i>et al.</i> (2008)
MSU III 18 (12)	SC	$S_1S_{13}'S_{36a}S_{36b3}$	TSUKAMOTO <i>et al.</i> (2008)
MSU 27e 9 (47)	SC	$S_{13}'S_{36a}S_{36b}S_x$	Our unpublished result
MSU 27 12 (54)	SI	$S_4S_6S_{36a}S_{36b}$	HAUCK <i>et al.</i> (2006b)
MSU 27 23 (16)	SI	$S_4S_6S_{36a}S_{36b}$	HAUCK <i>et al.</i> (2006b)
MSU 27 23 (42)	SI	$S_4S_6S_{36a}S_{36b}$	HAUCK <i>et al.</i> (2006b)
Sweet cherry			
Emperor Francis	SI	S_3S_4	Bošković and TOBUTT (2001)
Gold	SI	S_3S_6	HAUCK <i>et al.</i> (2001)
Hedelfingen	SI	S_3S_5	Bošković and TOBUTT (2001)
Rainier	SI	S_1S_4	Bošković and TOBUTT (2001)
Schmidt	SI	S_3S_4	Bošković and TOBUTT (2001)
Summit	SI	S_1S_2	Bošković and TOBUTT (2001)

^a Self-incompatible (SI) and self-compatible (SC).

^b S_{36a} , S_{36b} , and S_{36b2} were previously termed as S_a , S_b , and S_w respectively. S_{36b2} and S_{36b3} are mutated types of S_{36b} .

^c S_x is double dose for either S_{36a} or S_{36b} .

fosmid libraries were screened at 60° with a mixture of DIG-dUTP-labeled S_6 -*RNase* and *SFB*₆ probes, as previously described (USHIYAMA *et al.* 2001). The DIG-labeled S_6 -*RNase* and *SFB*₆ probes were obtained by PCR labeling using a PCR DIG probe synthesis kit (Roche) with Pru-C2 and PCE-R primers (TAO *et al.* 1999; YAMANE *et al.* 2001) and SFB-C1F (IKEDA *et al.* 2004a) and SFB-C4R primers (YAMANE *et al.* 2003c), respectively. The S haplotype of each positive fosmid clone was determined by PCR with the *S-RNase* consensus primer pair (Pru-C2 and PCE-R), which detects length differences for the second intron of the *S-RNase*. Positive clones were also checked for the presence of *SFB* using PCR with the *SFB* consensus primer pair (SFB-C1F and SFB-C2R) (IKEDA *et al.* 2004a). *S-RNase* and *SFB* allele-specific primer pairs were also used to verify the S haplotypes. Fosmid DNAs were prepared using the Wizard Plus Minipreps DNA purification kit (Promega, Madison, WI).

DNA sequencing: Sequencing was carried out at the Michigan State University Research Technology Support

Facility. The fosmid clones were sequenced by primer walking using the primers Pru-T2, Pru-C2, Pru-C2R (TSUKAMOTO *et al.* 2006), PCE-F (TSUKAMOTO *et al.* 2006), PCE-R, Pru-C4R, Pru-C5, S36ab322F, and S36ab366F (supporting information, Table S1) for the *S-RNases*, and SFB-C1F, SFB-C2R, SFB-C5F, and FB3R (IKEDA *et al.* 2005) for the *SFBs*. The flanking regions for the *S-RNases* of the S_{36} variants were sequenced using end-specific primers (Table S1). To determine the nucleotide sequences of the fosmid (FOS)-forward and FOS-reverse ends of the S_{36} variants fosmid clones, pCCIFOS-F, pCCIFOS-R, and T7 primers were used (Table S1). End-specific primer pairs for S_{36} fosmid clones were also designed to determine the overlaps between fosmid clones (Table S2).

PCR amplification: PCR was conducted in a volume of 25 µl containing 50 ng of template DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 200 µM dNTP, 0.8 µM each primer, and 0.625 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR program was 35 cycles of denaturation at 94° for 30 sec, an annealing step (see Table 2 for temperature) for

PcS36a-RNase.nt	agatattttctgaagtATGGTGACGTTGAAATCGTCACTCGCTTTCCTTGTTCTTGGCTTTGCTTCTTGTTCTTATTATGAGCACTGGTGTGtaggctg	90
PcS36b-RNase.nt	agatattttctgaagtATGGTGACGTTGAAATCGTCACTCGCTTTCCTTGTTCTTGGCTTTGCTTCTTGTTCTTATTATGAGCACTGGTGTGtaggctg	90
PcS36b2-RNase.nt	agatattttctgaagtATGGTGACGTTGAAATCGTCACTCGCTTTCCTTGTTCTTGGCTTTGCTTCTTGTTCTTATTATGAGCACTGGTGTGtaggctg	90
PcS36b3-RNase.nt	agatattttctgaagtATGGTGACGTTGAAATCGTCACTCGCTTTCCTTGTTCTTGGCTTTGCTTCTTGTTCTTATTATGAGCACTGGTGTGtaggctg	90
PcS36a-RNase.aa	M V T L K S S L A F L V L A F A L F L C F I M S T G D	27
PcS36b-RNase.aa	M V T L K S S L A F L V L A F A L F L C F I M S T G D	27
PcS36b2-RNase.aa	M V T L K S S L A F L V L A F A L F L C F I M S T G D	27
PcS36b3-RNase.aa	M V T L K S S L A F L V L A F A L F L C F I M S T G D	27
PcS36a-RNase.nt	cattacaactcttttgccttttatatcccatatataatgcataaattagcgattgcgtttttgtacttccattttatgttttagggaatattgtgagtgccgatg	195
PcS36b-RNase.nt	cattacaactcttttgccttttatatcccatatataatgcataaattagcgattgcgtttttgtacttccattttatgttttagggaatattgtgagtgccgatg	195
PcS36b2-RNase.nt	cattacaactcttttgccttttatatcccatatataatgcataaattagcgattgcgtttttgtacttccattttatgttttagggaatattgtgagtgccgatg	195
PcS36b3-RNase.nt	cattacaactcttttgccttttatatcccatatataatgcataaattagcgattgcgtttttgtacttccattttatgttttagggaatattgtgagtgccgatg	195
PcS36a-RNase.nt	aaatatcaggtaacgaaggacttgatctaaccgcaata--gtcttttgggaattatttttctgcaataagtcagattatcatcattggaagccaaaattatgttctt	298
PcS36b-RNase.nt	aaatatcaggtaacgaaggacttgatctaaccgcaata--gtcttttgggaattatttttctgcaataagtcagattatcatcattggaagccaaaattatgttctt	298
PcS36b2-RNase.nt	aaatatcaggtaacgaaggacttgatctaaccgcaata--gtcttttgggaattatttttctgcaataagtcagattatcatcattggaagccaaaattatgttctt	300
PcS36b3-RNase.nt	aaatatcaggtaacgaaggacttgatctaaccgcaata--gtcttttgggaattatttttctgcaataagtcagattatcatcattggaagccaaaattatgttctt	298
PcS36a-RNase.nt	tatacataaaaaaactcttatttttagataccatttctcttctacaatatataattttcgagGATCTTTATGACTATTTTCAATTGTCACAAATGGCCACCGCG	403
PcS36b-RNase.nt	tatacataaaaaaactcttatttttagataccatttctcttctacaatatataattttcgagGATCTTTATGACTATTTTCAATTGTCACAAATGGCCACCGCG	403
PcS36b2-RNase.nt	tatacataaaaaaactcttatttttagataccatttctcttctacaatatataattttcgagGATCTTTATGACTATTTTCAATTGTCACAAATGGCCACCGCG	405
PcS36b3-RNase.nt	tatacataaaaaaactcttatttttagataccatttctcttctacaatatataattttcgagGATCTTTATGACTATTTTCAATTGTCACAAATGGCCACCGCG	403
PcS36a-RNase.aa	G S Y D Y F Q F V Q Q W P P A	42
PcS36b-RNase.aa	G S Y D Y F Q F V Q Q W P P A	42
PcS36b2-RNase.aa	G S Y D Y F Q F V Q Q W P P A	42
PcS36b3-RNase.aa	G S Y D Y F Q F V Q Q W P P A	42
PcS36a-RNase.nt	TACCTGCAGCTCTTAGCAGGACACCTTGCTACAAACCCCGGCCACCTCAAATCTTACCATCCATGGCCATAGGCCAAGTAACATTCAAACCCAAAGAGGCCAG	508
PcS36b-RNase.nt	TACCTGCAGCTCTTAGCAGGACACCTTGCTACAAACCCCGGCCACCTCAAATCTTACCATCCATGGCCATAGGCCAAGTAACATTCAAACCCAAAGAGGCCAG	508
PcS36b2-RNase.nt	TACCTGCAGCTCTTAGCAGGACACCTTGCTACAAACCCCGGCCACCTCAAATCTTACCATCCATGGCCATAGGCCAAGTAACATTCAAACCCAAAGAGGCCAG	510
PcS36b3-RNase.nt	TACCTGCAGCTCTTAGCAGGACACCTTGCTACAAACCCCGGCCACCTCAAATCTTACCATCCATGGCCATAGGCCAAGTAACATTCAAACCCAAAGAGGCCAG	508
PcS36a-RNase.aa	T C S L S R T P C Y K P R P P Q I F T I H G L W P S N Y S N P K R P S	77
PcS36b-RNase.aa	T C S L S R T P C Y K P R P P Q I F T I H G L W P S N Y S N P K R P S	77
PcS36b2-RNase.aa	T C S L S R T P C Y K P R P P Q I F T I H G L W P S N Y S N P K R P S	65
PcS36b3-RNase.aa	T C S L S R T P C Y K P R P P Q I F T I H G L W P S N Y S N P K R P S	77
PcS36a-RNase.nt	TAATTGACAGGGGGTCGCTATTGATTCAAGGAAAGTGgtatgtatgtatttttttctcacttactcttttagcatttagttttaa--aaaagtttagattgtcatat	612
PcS36b-RNase.nt	TAATTGACAGGGGGTCGCTATTGATTCAAGGAAAGTGgtatgtatgtatttttttctcacttactcttttagcatttagttttaa--aaaagtttagattgtcatat	612
PcS36b2-RNase.nt	TAATTGACAGGGGGTCGCTATTGATTCAAGGAAAGTGgtatgtatgtatttttttctcacttactcttttagcatttagttttaa--aaaagtttagattgtcatat	615
PcS36b3-RNase.nt	TAATTGACAGGGGGTCGCTATTGATTCAAGGAAAGTGgtatgtatgtatttttttctcacttactcttttagcatttagttttaa--aaaagtttagattgtcatat	612
PcS36a-RNase.aa	N C R G S L F D S R K V	89
PcS36b-RNase.aa	N C R G S L F D S R K V	89
PcS36b3-RNase.aa	N C R G S L F D S R K V	89
PcS36a-RNase.nt	gaagacagtagacactctttcgataaaaactctgggtgttagataaaactctgatgttgcctttatcccaacatagggggaggagggtgtgtatgttaggaaattct	717
PcS36b-RNase.nt	gaagacagtagacactctttcgataaaaactctgggtgttagataaaactctgatgttgcctttatcccaacatagggggaggagggtgtgtatgttaggaaattct	717
PcS36b2-RNase.nt	gaagacagtagacactctttcgataaaaactctgggtgttagataaaactctgatgttgcctttatcccaacatagggggaggagggtgtgtatgttaggaaattct	718
PcS36b3-RNase.nt	gaagacagtagacactctttcgataaaaactctgggtgttagataaaactctgatgttgcctttatcccaacatagggggaggagggtgtgtatgttaggaaattct	717
PcS36a-RNase.nt	ctccactcttttttcttgatacaagggggaggagggtgagccttaggacatcggtgcataagataaagccttaactactattttgaaagtccttcttctt	822
PcS36b-RNase.nt	ctccactcttttttcttgatacaagggggaggagggtgagccttaggacatcggtgcataagataaagccttaactactattttgaaagtccttcttctt	822
PcS36b2-RNase.nt	ctccactcttttttcttgatacaagggggaggagggtgagccttaggacatcggtgcataagataaagccttaactactattttgaaagtccttcttctt	823
PcS36b3-RNase.nt	ctccactcttttttcttgatacaagggggaggagggtgagccttaggacatcggtgcataagataaagccttaactactattttgaaagtccttcttctt	822
PcS36a-RNase.nt	aattttaagtttttctcacttttataatttttattttcataagataaaaaaataagtaatttagtccagctgtgaaaaataaattttttttttaaggtggacacatgacc	927
PcS36b-RNase.nt	aattttaagtttttctcacttttataatttttattttcataagataaaaaaataagtaatttagtccagctgtgaaaaataaattttttttttaaggtggacacatgacc	927
PcS36b2-RNase.nt	aattttaagtttttctcacttttataatttttattttcataagataaaaaaataagtaatttagtccagctgtgaaaaataaattttttttttaaggtggacacatgacc	928
PcS36b3-RNase.nt	aattttaagtttttctcacttttataatttttattttcataagataaaaaaataagtaatttagtccagctgtgaaaaataaattttttttttaaggtggacacatgacc	927
PcS36a-RNase.nt	atattttaagttgggagtagcattattgtttacatatttttatgttaatacaaaatcactaattcagtagtagtactcagggttaacgtgaaaaaattatcttattcaa	1032
PcS36b-RNase.nt	atattttaagttgggagtagcattattgtttacatatttttatgttaatacaaaatcactaattcagtagtagtactcagggttaacgtgaaaaaattatcttattcaa	1032
PcS36b2-RNase.nt	atattttaagttgggagtagcattattgtttacatatttttatgttaatacaaaatcactaattcagtagtagtactcagggttaacgtgaaaaaattatcttattcaa	1033
PcS36b3-RNase.nt	atattttaagttgggagtagcattattgtttacatatttttatgttaatacaaaatcactaattcagtagtagtactcagggttaacgtgaaaaaattatcttattcaa	1032
PcS36a-RNase.nt	gaacggaaatctctctctttaagtttttactattctttaaataatgtatgaattgtgttgagtgctcagtagtaccctgaggttgcggaattgcaattcgaagatattcttgcg	1137
PcS36b-RNase.nt	gaacggaaatctctctctttaagtttttactattctttaaataatgtatgaattgtgttgagtgctcagtagtaccctgaggttgcggaattgcaattcgaagatattcttgcg	1137
PcS36b2-RNase.nt	gaacggaaatctctctctttaagtttttactattctttaaataatgtatgaattgtgttgagtgctcagtagtaccctgaggttgcggaattgcaattcgaagatattcttgcg	1136
PcS36b3-RNase.nt	gaacggaaatctctctctttaagtttttactattctttaaataatgtatgaattgtgttgagtgctcagtagtaccctgaggttgcggaattgcaattcgaagatattcttgcg	1135
PcS36a-RNase.aa	Y P Q L R L N L K I S W	101
PcS36b-RNase.aa	Y P Q L R L N L K I S W	101
PcS36b3-RNase.aa	Y P Q L R L N L K I S W	101
PcS36a-RNase.nt	CCAAGCTGAAAGTGGCAATGATACAGAAATTTTGGGAAAGCGAATGGAACAAACATGGTAGATGTTCCGACACAGACACTCAACCAAAATGCAATACCTTCGAGCGAT	1242
PcS36b-RNase.nt	CCAAGCTGAAAGTGGCAATGATACAGAAATTTTGGGAAAGCGAATGGAACAAACATGGTAGATGTTCCGACACAGACACTCAACCAAAATGCAATACCTTCGAGCGAT	1242
PcS36b2-RNase.nt	CCAAGCTGAAAGTGGCAATGATACAGAAATTTTGGGAAAGCGAATGGAACAAACATGGTAGATGTTCCGACACAGACACTCAACCAAAATGCAATACCTTCGAGCGAT	1241
PcS36b3-RNase.nt	CCAAGCTGAAAGTGGCAATGATACAGAAATTTTGGGAAAGCGAATGGAACAAACATGGTAGATGTTCCGACACAGACACTCAACCAAAATGCAATACCTTCGAGCGAT	1240
PcS36a-RNase.aa	P N V K S G N D T E F W E S E W N K H G R C S E Q T L N Q M Q Y F E R	136
PcS36b-RNase.aa	P N V K S G N D T E F W E S E W N K H G R C S E Q T L N Q M Q Y F E R	136
PcS36b3-RNase.aa	P N V K S G N D T E F W E S E W N K H G R C S E Q T L N Q M Q Y F E R	136
PcS36a-RNase.nt	CAGACGAAATGTGGAACCTTTACAATATTACGGAGATCTTAAAAAGCTCAAAATCGTACCAAAATGCGACACGGACATGGAAGTACTCGGACATATTATCACCCA	1347
PcS36b-RNase.nt	CAGACGAAATGTGGAACCTTTACAATATTACGGAGATCTTAAAAAGCTCAAAATCGTACCAAAATGCGACACGGACATGGAAGTACTCGGACATATTATCACCCA	1347
PcS36b2-RNase.nt	CAGACGAAATGTGGAACCTTTACAATATTACGGAGATCTTAAAAAGCTCAAAATCGTACCAAAATGCGACACGGACATGGAAGTACTCGGACATATTATCACCCA	1346
PcS36b3-RNase.nt	CAGACGAAATGTGGAACCTTTACAATATTACGGAGATCTTAAAAAGCTCAAAATCGTACCAAAATGCGACACGGACATGGAAGTACTCGGACATATTATCACCCA	1345
PcS36a-RNase.aa	S D E M W N S Y N I T E I L K K A Q I V P N A T R T W K Y S D I L S P	171
PcS36b-RNase.aa	S D E M W N S Y N I T E I L K K A Q I V P N A T R T W K Y S D I L S P	171
PcS36b3-RNase.aa	S D E M W N S Y N I T E I L K K A Q I V P N A T R T W K Y S D I L S P	171
PcS36a-RNase.nt	TTAAAGCAGCAACTAACCAACACCCATCTCTTGGTGAACACCTGATCAGCAGCTTAAGAGCCAAACCATCGCAGCCTAAGAGCCCCCAAAGGCTCAGTTGT	1452
PcS36b-RNase.nt	TTAAAGCAGCAACTAACCAACACCCATCTCTTGGTGAACACCTGATCAGCAGCTTAAGAGCCAAACCATCGCAGCCTAAGAGCCCCCAAAGGCTCAGTTGT	1452
PcS36b2-RNase.nt	TTAAAGCAGCAACTAACCAACACCCATCTCTTGGTGAACACCTGATCAGCAGCTTAAGAGCCAAACCATCGCAGCCTAAGAGCCCCCAAAGGCTCAGTTGT	1451
PcS36b3-RNase.nt	TTAAAGCAGCAACTAACCAACACCCATCTCTTGGTGAACACCTGATCAGCAGCTTAAGAGCCAAACCATCGCAGCCTAAGAGCCCCCAAAGGCTCAGTTGT	1450
PcS36a-RNase.aa	I K A A T N T T P I L R C K P D P A Q S K S Q P S Q P K S P Q K P Q L	206
PcS36b-RNase.aa	I K A A T N T T P I L R C K P D P A Q S K S Q P S Q P K S P Q K P Q L	206
PcS36b3-RNase.aa	I K A A T N T T P I L R C K P D P A Q S K S Q P S Q P K S P Q K P Q L	206
PcS36a-RNase.nt	TACATGAAGTGGTATTTTGTTATGATTATCATGCAAAAAAGCAGATTGACTGTAACCGAACAGGATGCCTTAATAAAGACATCTCGTTTCAATAAaattatagct	1557
PcS36b-RNase.nt	TACATGAAGTGGTATTTTGTTATGATTATCATGCAAAAAAGCAGATTGACTGTAACCGAACAGGATGCCTTAATAAAGACATCTCGTTTCAATAAaattatagct	1557
PcS36b2-RNase.nt	TACATGAAGTGGTATTTTGTTATGATTATCATGCAAAAAAGCAGATTGACTGTAACCGAACAGGATGCCTTAATAAAGACATCTCGTTTCAATAAaattatagct	1556
PcS36b3-RNase.nt	TACATGAAGTGGTATTTTGTTATGATTATCATGCAAAAAAGCAGATTGACTGTAACCGAACAGGATGCCTTAATAAAGACATCTCGTTTCAATAAaattatagct	1555
PcS36a-RNase.aa	L H E V V F C Y D Y H A K K Q I D C N R T G C L N K D I S F Q *	237
PcS36b-RNase.aa	L H E V V F C Y D Y H A K K Q I D C N R T G C L N K D I S F Q *	237
PcS36b3-RNase.aa	L H E V V F C Y D Y H A K K Q I D C N R T G C L N K D I S F Q *	237

30 sec, and extension at 72° (see Table 2 for the extension time), with an initial denaturing step at 94° for 2 min 30 sec and a final extension at 72° for 7 min. To discriminate *S*_{36b2} from other *S*₃₆ variants, the derived cleaved amplified polymorphic sequence (dCAPS) primer (NEFF *et al.* 1998), AvaS36b2-F (Table 2), was designed using dCAPS finder 2.0 (NEFF *et al.* 2002). Five microliters of PCR products was digested for 4 hr at 37° in 20 µl total volume with 3 units of the appropriate restriction endonuclease. PCR products and digested PCR products were resolved on 2.0–3.0% agarose gels and stained with ethidium bromide.

RT-PCR: Total RNA was isolated from styles at the balloon stage of flower development and from pollen grains as described elsewhere (TAO *et al.* 1999). Total RNA was treated with DNaseI (Roche) to avoid contamination of genomic DNA before being used for all RT-related studies. One microgram of RNA was used for first-strand cDNA synthesis by SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen) with oligo(dT) primer following the manufacturer's protocol. For *S-RNase* expression, allele-specific primers, PcS36ab-RNase-F and PcS36ab-RNase-R (Table 2) were used to amplify the four *S*₃₆ *RNase* variants. PCR conditions were identical to those described by TAO *et al.* (1999). For *SFB* expression, allele-specific primers, PcSFB36ab-F and PcSFB36ab-R (Table 2) were used to amplify the four *SFB*₃₆ variants. Total pollen RNA was used as a negative control to confirm the absence of contamination from genomic DNA. Expression of the *actin* gene was used as a reference with the primers ActF1 and ActR1 (YAMANE *et al.* 2003a). PCR conditions were identical to those described by USHIJIMA *et al.* (2004). PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

3' RACE of *SFB*₃₆ variants: One microgram of pollen RNA from the sour cherry selections MSU 27e 9 (47) and Tamaris (Table 1) was used for first-strand cDNA synthesis by SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen) with the oligo(dT)-adaptor primer (5'-TTTTTTTTTTTTTTTGGACCGGCAGCAAATGTTGCAGC-3') following the manufacturer's protocol. PcSFB36ab-F and the adaptor primer (5'-TGACCGGCAGCAAATGTTGCAGC-3') were used to amplify the two *SFB*₃₆ variants (*SFB*_{36a} and/or *SFB*_{36b}). The resulting PCR products were recovered from the gel using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and ligated into the pJET1.2 vector using the CloneJET PCR cloning kit (Fermentas, Burlington, Canada). Colony PCR was performed for 13 clones with the PcSFB36ab-F and PcSFB36ab-R primers, and the PCR products were digested with *Rsa*I to distinguish *SFB*_{36a} clones from *SFB*_{36b} clones. One clone for each *SFB*₃₆ variant was sequenced using pJET1.2 vector-specific primers that were supplied with the CloneJET PCR cloning kit (Fermentas). Two *SFB*₃₆ allele-specific primers, PcSFB36ab-693F and PcSFB36ab-845F, were also used for sequencing (Table S1).

RT-PCR of poly(A)⁺ pollen RNA: Seven micrograms of pollen RNA of MSU 27e 9 (47) was separated into poly(A)⁺ and poly(A)⁻ pollen RNA using the Oligotex mRNA mini kit (Qiagen). One hundred nanograms of poly(A)⁺ and poly(A)⁻ pollen RNA were used for first-strand cDNA synthesis by SuperScript II RNase H⁻ reverse transcriptase (Invitrogen). First-strand cDNAs of the *SFB*₃₆ variants, *SFB*_{13'}, and the *actin* gene were synthesized using the PcSFB36ab-R, PcSFB13-R (TSUKAMOTO *et al.* 2006), and ActR1 primers, respectively.

Amplification of the *SFB*₃₆ variants, *SFB*_{13'}, and the *actin* genes utilized the primer pairs PcSFB36ab-F and PcSFB36ab-R, PcSFB13-F (TSUKAMOTO *et al.* 2006) and PcSFB13-R, and ActF1 and ActR1, respectively. PCR products were cloned and sequenced as described above.

RESULTS

Molecular characterization of the four *S*₃₆ haplotype variants: To determine whether the *S*_{36a} and *S*_{36b} haplotypes had complementary alterations in *S-RNase* and *SFB*, these genes were cloned from fosmid libraries generated from two SC cultivars: Rheinische Schattenmorelle (RS) (*S*₆*S*_{13'}*S*₂₆*S*_{36a}) and Újfehértói fűrtös (UF) (*S*_{1'}*S*₄*S*₃₅*S*_{36b}), respectively. The nucleotide sequences of the relevant clones revealed that the *S*_{36a} *RNase* differed from that of the *S*_{36b} *RNase* by one nucleotide mismatch in the second intron (Figure 1). *SFB*_{36a} and *SFB*_{36b} differed by eight nucleotides and their deduced amino acids had only five amino acid differences (Figure 2). The high sequence similarity between the *S*_{36a} and *S*_{36b} haplotypes suggests that they may be derived from the same ancestral S haplotype.

A comparison of the *S*_{36a} and *S*_{36b} *RNases* with other functional cherry *S-RNases* did not reveal any molecular alterations that are known to disrupt *S-RNase* activity (Figure S1). However, the amino acid sequences of the *S*_{36a} and *S*_{36b} *RNases* were longer than the majority of the other functional cherry *S-RNases* in the region just before the conserved C5 region (Figure S1). It is unlikely that the length is the cause of the loss of function of the *S*_{36a} and *S*_{36b} *RNases* because the sour cherry *S*₃₃ *RNase*, whose amino acid sequence is one residue longer than the *S*_{36a} and *S*_{36b} *RNases* in this hypervariable region, is functional (TSUKAMOTO *et al.* 2008).

The amino acid sequences of *SFB*_{36a} and *SFB*_{36b} were compared with other functional cherry *SFBs* (Figure 2 and Figure S2). All the functional cherry *SFBs* identified to date have a conserved lysine (K), a conserved tyrosine (Y), and a conserved glycine (G) at positions 53, 188, and 214, respectively. In contrast, both *SFB*_{36a} and *SFB*_{36b} have isoleucine (I), phenylalanine (F), and serine (S) residues at positions 53, 188, and 214, respectively. Although there are three conserved amino acids in cherry, only one of them, the tyrosine residue at position 188 (Figure S3), is conserved in all known functional *SFBs* from *Prunus* species. This amino acid may thus be critical for the function (see DISCUSSION).

Because the sequence analysis of the *S-RNase* and *SFB* for the *S*_{36a} and *S*_{36b} haplotypes failed to identify any

FIGURE 1.—Nucleotide and amino acid alignments of the *S-RNases* from the four *S*₃₆ haplotypes. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering, Tokyo). Gaps are marked by dashes. Adenine of the start codon was positioned to be +1. The region from -1 to -15 shown in lowercase is the 5' flanking region. The intron sequences are indicated by lowercase letters, whereas exon sequences are shown in uppercase letters. Conserved nucleotides are shown on a darkened background. The five conserved regions—C1, C2, C3, RC4, and C5 (USHIJIMA *et al.* 1998)—are marked with solid boxes, and the hypervariable region, RHV (USHIJIMA *et al.* 1998), reported in the rosaceous *S-RNases*, is marked with a dotted box.

FIGURE 2.—Nucleotide and amino acid alignments of the SFBs from the *S*_{36a} and *S*_{36b} haplotypes as the SFBs from *S*_{36b2} and *S*_{36b3} are identical to SFB_{36b}. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering, Tokyo). Adenine of the start codon was positioned to be +1. The region from -1 to -15 shown is the 5' flanking region. Conserved nucleotides are shown on a darkened background. The locations of the F-box motif—V1, V2, HvA and HvB (IKEDA *et al.* 2004a), and Vn (NUNES *et al.* 2006)—are indicated by solid boxes. The asterisks under the amino acid sequence indicate the five amino acid residues that differ between SFB_{36a} and SFB_{36b}. The positions and directions of the primers used in genomic PCR to specifically amplify SFB_{36a} and SFB_{36b} are indicated by arrows.

molecular alternation that could be conclusively linked to a loss of specificity function, we compared the regions flanking the two specificity genes. These two haplotypes were already known to have a differential *Hind*III cut site yielding RFLP products that differ by ~200 bp (Figure 3) (YAMANE *et al.* 2001). In total, 7829 and 8217 bp of the upstream and downstream flanking regions of the *S*-RNase of *S*_{36a} and *S*_{36b} haplotypes, respectively, were determined through multiple rounds of primer walking (Figure 4; see Figure S4 and Table S1 for the fosmid clones and primers used for sequencing). The *S*_{36a} and *S*_{36b} sequences each had one conserved *Hind*III

cut site at positions 2137/2138 and 2139/2140 bp upstream from the start codon of the *S*_{36a} RNase and *S*_{36b} RNase, respectively (Figure 4 and Figure S5). The sequences had high identity (98.6%) from the *S*-RNase start codon to position ~2660 bp (in red in Figure 4). However, downstream from position 2660 bp, these two *S* haplotypes had no significant sequence identity (24.2% or not alignable, in green and aqua in Figure 4), and they had a second and differential *Hind*III site downstream of the *S*-RNase start codon that accounted for the polymorphism between the *S*_{36a} haplotype and the *S*_{36b} haplotype (Figure 4 and Figure S6). The *Hind*III

FIGURE 3.—Southern blot analysis. Genomic DNAs digested with *Hind*III were hybridized with the *S*₆ *RNase* cDNA probe radiolabeled with [³²P]dCTP. Circles, asterisks, and an arrowhead indicate the *S* haplotype-specific fragment of *S*_{36a} (6.1 kb), *S*_{36b} (5.8 kb), and *S*_{36b3} (4.8 kb), respectively. Lane abbreviations are the following: UF, Újfehértói fürtös; EB, Erdi Botermo; RS, Rheinische Schattenmorelle; Mont, Montmorency; III 18 (12), MSU III 18 (12); ET, Englaise Timpurii.

site in *S*_{36a} was at 3934/3935 bp downstream from the *S*_{36a} *RNase* start codon, whereas the *Hind*III site in *S*_{36b} was at 3668/3669 bp downstream from the *S*_{36b} *RNase* start codon. This differential cut site resulted in the different *Hind*III fragment sizes for the *S*_{36a} *RNase* and *S*_{36b} *RNase*: 6071 and 5807 bp, respectively.

Identification and characterization of the *S*_{36b2} haplotype: A common forward primer (PcS36ab-F, located in the homologous sequence downstream of the *S*_{36a} and *S*_{36b} *RNases*) and two *S* haplotype-specific reverse primers (PcS36a-spR and PcS36b/b2/b3-R, located ~3400 bp downstream from the *S*-*RNase* start codon) were designed to distinguish between the *S*_{36a}

and *S*_{36b} haplotypes (Table 2 and Figure 4). Fragment sizes of 898 and 760 bp were amplified from the *S*_{36a} and *S*_{36b} haplotypes (Figure 5, A and B), respectively. When these primers were tested against an array of sour cherry selections, the *S* genotypes for two selections contradicted our previous results. Cigány 59 and Tschernokorka were reported to have the *S*_{36a} (*S*_a) haplotype (YAMANE *et al.* 2001); however, the *S*_{36a}-specific primers failed to amplify a fragment from these cultivars. Instead, the *S*_{36b}-specific primers amplified a fragment of the expected size (Figure 5, A and B). To further investigate the *S*₃₆ haplotypes in Cigány 59 and Tschernokorka, a fosmid library from Cigány 59 was generated and screened to identify the *S*-*RNase* and *SFB*. These two genes were sequenced from three Cigány 59 *S*₃₆ clones.

The *S*₃₆ *RNase* variant from Cigány 59 differed from the *S*_{36b} *RNase* by a 1-bp substitution (G/A) in the conserved region C2, causing a premature stop codon (Figure 1) and thereby providing an explanation for the loss of function for this unique *S*₃₆ haplotype. This additional *S*₃₆ haplotype variant in Cigány 59 was named *S*_{36b2}. The flanking regions of the *S*_{36b2} *RNase* were sequenced, revealing a 307-bp insertion between 752 and 1058 bp upstream of the *S*_{36b2} *RNase* that was not present in the upstream regions of the *S*_{36b} *RNase* of UF or the *S*_{36a} *RNase* of RS (Figure 4 and Figure S5). Therefore, after *Hind*III digestion, the size of the fragment containing the *S*_{36b2} *RNase* is 6097 bp, close to the length (6071 bp) of the *S*_{36a} *RNase*. This explains why the *S*_{36b2} of Cigány 59 was thought to be *S*_{36a} (*S*_a) by YAMANE *et al.* (2001). The nucleotide sequence of the *SFB* obtained from the *S*_{36b2} haplotype was identical to that from the *S*_{36b} haplotype; therefore, we also used the nomenclature *SFB*_{36b} to refer to the *SFB* from the *S*_{36b2} haplotype.

Identification and characterization of the *S*_{36b3} haplotype: Another sour cherry selection, MSU III 18 (12), also tested positive for the presence of the *S*_{36b} haplotype by PCR with *S*_{36b}-specific primers (Figure 5B); however, MSU III 18 (12) did not have the *S*_{36b} *RNase* *Hind*III fragment (5.8 kb). Instead, the observed *Hind*III fragment (4.8 kb, Figure 3) was smaller than expected for the *S*_{36b} haplotype (5.8 kb), suggesting that MSU III 18 (12) might contain an additional variant of the *S*_{36b} haplotype, *S*_{36b3}. To investigate this possibility, a fosmid library was generated from MSU III 18 (12) for molecular analysis of the *S*_{36b3} haplotype. Three clones, each containing the *S*-*RNase* and *SFB* of the *S*_{36b3} haplotype, were obtained and sequenced. The amino acid sequence of the *S*-*RNase* was identical to that of *S*_{36b} (Figure 1); however, the second intron exhibited five nucleotide differences and one indel (Figure 1). Upon sequencing the regions flanking the *S*_{36b3} *RNase* of MSU III 18 (12), a 1-bp substitution (A/G) was identified at 2727 bp downstream of the *S*_{36b3} *RNase*. This substitution led to another *Hind*III cut site between 2725 and 2726 bp (Figure 4 and Figure S6) and therefore explains

FIGURE 4.—Diagram of the molecular differences of the *S* locus for four *S*₃₆ variants. Red and blue boxes indicate the *S*-*RNase* and *SFB*, respectively. The positions and directions of the primers used in sequencing are indicated by red, green, and aqua arrows. The positions and directions of the primers used in genomic PCR are indicated by black arrows. The red regions are conserved among the four *S*₃₆ variants. The green region was specific to the *S*_{36a} haplotype whereas the aqua region was conserved among *S*_{36b}, *S*_{36b2}, and *S*_{36b3}. The 307-bp insertion in *S*_{36b2} is indicated by purple.

why the *Hind*III digestion fragment from MSU III 18 (12) was 4871 bp (Figure 4). The *SFB* nucleotide sequence was identical to that of the *S*_{36b} haplotype. As the nucleotide sequences of the *SFB*s from both the *S*_{36b} and the *S*_{36b3} haplotype were identical, we used the nomenclature *SFB*_{36b} to refer to both.

Development of molecular markers to distinguish among the *S*₃₆ variants: The *S*_{36b}/*S*_{36b2}/*S*_{36b3}-specific primer pair, PcS36ab-F and PcS36b/b2/b3-R, amplified an ~760-bp product of the expected size for *S*_{36b}, *S*_{36b2}, and *S*_{36b3} (Figure 5B). Since the PCR product from MSU III 18 (12) contained an additional *Hind*III site, this primer pair works as a cleaved amplified polymorphic sequence (CAPS) (KONIECZNY and AUSUBEL 1993) marker to distinguish *S*_{36b3} from *S*_{36b} and *S*_{36b2}. The PCR product of *S*_{36b3} was cut by *Hind*III into two fragments of 585 and 175 bp, whereas those of *S*_{36b} and *S*_{36b2} were uncut (Figure 5G).

To test for the presence of *S*_{36b2} and *S*_{36b} in different cherry cultivars, primers were designed to differentiate *S*_{36b2} from *S*_{36b}. The primer pair PcS36ab-F2 and PcS36ab-R was designed to flank the region of the 307-bp insert (Table 2 and Figure 4). This primer pair amplified an ~1000-bp product of the expected size (1042 bp) from *S*_{36a}, *S*_{36b}, and *S*_{36b3} whereas a product of the expected size (1348 bp) was amplified from *S*_{36b2} (Figure 5C). An insert-specific primer, PcS36b2-spR, was also designed, which, together with the forward primer PcS36ab-F2, amplified a product of the expected size (513 bp) for *S*_{36b2} but not for *S*_{36a}, *S*_{36b}, and *S*_{36b3} (Figure 5D). The dCAPS primer AvaS36b2-F was also designed based upon the 1-bp substitution in the C2 region (Figure 5F). The primer AvaS36b2-F and the newly designed S36ab-RNase-specific primer, PcS36ab-RNase-R2 (Table 2), amplified 274-, 271-, 273-, and 274-bp products for *S*_{36a}, *S*_{36b}, *S*_{36b2}, and *S*_{36b3}, respectively (Figure 5E). After digestion with

TABLE 2

PCR primer pairs designed to discriminate between the mutated S haplotypes and their wild types

S haplotype Primer name	Primer sequence 5' → 3'	Annealing temperature	Extension time (s)	S haplotype	Product size (bp)	Enzyme	Fragment size (bp) ^a
<i>S</i> ₃₆ RNase consensus primer							
PcS36ab-RNase-F	CCGGCTACCTGCAGTCTTAG	66	60	<i>S</i> _{36a} , <i>S</i> _{36b}	760	—	—
PcS36ab-RNase-R	CATTGCCACTTTTCACGTTG			<i>S</i> _{36b2} , <i>S</i> _{36b3}			
<i>SFB</i> ₃₆ consensus primer							
PcSFB36ab-F	GGCGGTCGATCCTGATGAC	62	60	<i>S</i> _{36a}	721	<i>Rsa</i> I	326/262/133
PcSFB36ab-R	TGTCCGATAACAGCTCCGG			<i>S</i> _{36b} , <i>S</i> _{36b2} , <i>S</i> _{36b3}	721	<i>Rsa</i> I	326/284/111
<i>S</i> _{36a} -specific primer							
PcS36ab-F	GCTAGCCAACCACTTTTACG	66	60	<i>S</i> _{36a}	898	—	—
PcS36a-spR	GAAACCCACATGATACAAACTG						
<i>S</i> _{36b} / <i>S</i> _{36b2} / <i>S</i> _{36b3} -specific primer							
PcS36ab-F	GCTAGCCAACCACTTTTACG	66	60	<i>S</i> _{36b}	760	<i>Hind</i> III	760
PcS36b/b2/b3-R	ATACATTGTAGGCCAGTCTGTG			<i>S</i> _{36b2}	759	<i>Hind</i> III	759
				<i>S</i> _{36b3}	760	<i>Hind</i> III	585/175
<i>S</i> _{36b2} vs. <i>S</i> _{36a} / <i>S</i> _{36b} / <i>S</i> _{36b3}							
PcS36ab-F2	CTCTCATCTCTCCCTTTGTTG	66	75	<i>S</i> _{36a} , <i>S</i> _{36b}	1042	—	—
PcS36ab-R	GGGTGTACTATCGCAATATCTC			<i>S</i> _{36b3} <i>S</i> _{36b2}	1348	—	—
<i>S</i> _{36b2} insert-specific primer							
PcS36ab-F2	CTCTCATCTCTCCCTTTGTTG	67	60	<i>S</i> _{36b2}	513	—	—
PcS36b2-spR	CCACCACCTCAAAAATGTTATA						
<i>S</i> _{36b2} dCAPS							
AvaS36b2-F	CTTCACCATCCATGGCCTAGG	62	20	<i>S</i> _{36a} , <i>S</i> _{36b3}	274	<i>Ava</i> II	274
PcS36ab-RNase-R2	AAAGAGTGGAGAGAATTCCTAAC			<i>S</i> _{36b}	271	<i>Ava</i> II	271
				<i>S</i> _{36b2}	273	<i>Ava</i> II	253/20

^a Product size is based on the use of genomic DNA as a template.

AvaII, only the PCR product obtained from the *S_{36b2}* haplotype was cut into 253- and 20-bp fragments (Figure 5H). Using these primers, Tschernokorka was determined to also have *S_{36b2}* (Figure 5, C–E and H).

Expression of *S-RNase* and *SFB* for the *S₃₆* variants:

To test whether the *S-RNase* and *SFB* from the *S₃₆* variants were transcribed, RT-PCR was preformed. For the *S_{36a}* *RNase*, the expected 760-bp fragment was amplified from Montmorency genomic DNA (Figure 6A). Smaller fragments (~210 bp) for the *S_{36a}* and *S_{36b}* *RNases*, which reflect the splicing of the second intron (555 bp), were obtained from cDNAs from Montmorency (*S₆S_{13m}S₃₅S_{36a}*), RS (*S₆S₁₃'S₂₆S_{36a}*), and UF (*S₁'S₄S₃₅S_{36b}*). Likewise, the expected fragment corresponding to *SFB_{36a}* was amplified from Montmorency (with *S_{36a}*), and the expected fragments corresponding to *SFB_{36b}* were amplified from Pandey 114 (*S₁S₄S₃₅S_{36b}*), UF (*S₁'S₄S₃₅S_{36b}*), and Cigány 59 (*S_{6m2}S₉S₂₆S_{36b2}*) (Figure 6B). These results suggested that, despite the nonfunctionality of the *S_{36a}* and *S_{36b}* haplotypes, both *S-RNase* and *SFB* are transcribed.

In the four *S₃₆* variants described, a small non-autonomous *Helitron* element was identified 38 bp downstream of the *SFB* stop codon. This element is 306 bp in

length and contains a GC-rich stem loop, which is typical for *Helitron* elements (KAPITONOV and JURKA 2001). The GC-rich stem loop is followed by an AT-rich region with the sequence TTTTTTTAAAAATAAAA, in which AAT AAA is the putative poly(A) signal. To test whether the insertion of this element impacts transcription termination or polyadenylation, 3' RACE was performed for *SFB_{36a}* from MSU 27e 9 (47) (*S₁₃'S_{36a}S_{36b}S_x*) and for *SFB_{36b}* from both MSU 27e 9 (47) and Tamaris (*S₁₃'S₁₆S₃₄S_{36b2}*). The ~950-bp RT-PCR products (data not shown) that were amplified with an adaptor primer and PcSFB36ab-F located within the *SFB* were cloned. To distinguish *SFB_{36a}* clones from *SFB_{36b}* clones, colony PCR was performed with PcSFB36ab-F and PcSFB36ab-R. Since the PCR products from *SFB_{36a}* and *SFB_{36b}* contain different *RsaI* sites, *RsaI* digestion was used to distinguish these two *SFBs* (Table 2). Six and five clones for *SFB_{36a}* and *SFB_{36b}*, respectively, were obtained from MSU 27e 9 (47), and two *SFB_{36b}* clones were obtained from Tamaris (Figure S7). Among them, 27e 9 (47)-8, 27e 9 (47)-3, and Tamaris-1 were selected for *SFB_{36a}*, *SFB_{36b}*, and *SFB_{36b}*, respectively, and their sequences were determined. The poly(A) tail was located 11 bp downstream of the putative stop codon in 27e 9 (47)-8 and

FIGURE 5.—PCR differentiation among the S_{36} haplotypes. (A) PCR with PcS36ab-F and PcS36a-spR. (B) PCR with PcS36ab-F and PcS36b/b2/b3-R. (C) PCR with PcS36ab-F2 and PcS36ab-R. (D) PCR with PcS36ab-F2 and PcS36b2-spR. (E) PCR with AvaS36b2-F and PcS36ab-RNase-R2. (F) Nucleotide sequences of S_{36} variants used to design dCAPS marker AvaS36b2-F. (G) PCR fragments from B digested with *Hind*III. (H) PCR fragments digested with *Ava*II. Lane abbreviations are the following: C59, Cigány 59; Cri, Crisana; ET, Englaise Timpurii; EB, Erdi Botermo; EJ, Erdi Jubileum; EN, Erdi Nagygy-molcsu; Met, Meteor; Mon, Montmorency; P38, Pandi 38; P114, Pandi 114; RS, Rheinische Schattenmorelle; Sur, Surefire; Tar, Tarina; Tsc, Tschernokorka; UF, Újfehértói fürtös; III 18 (12), MSU III 18 (12).

Tamaris-1 and 34 bp downstream of the stop codon in 27e 9 (47)-3 (Figure S8). This suggests that at least some of the SFB_{36} mRNAs are polyadenylated, and the mature mRNAs end before the *Helitron* element.

To investigate whether a short distance between the stop codon and the poly(A) tail might affect *SFB* function, we examined *SFB* mRNA sequences derived from *Prunus* species that are available in GenBank. Twelve nonredundant mRNA sequences were retrieved and the distance between the poly(A) tail and the stop codon ranged from 11 to 318 bp. Notably, the poly(A) tail

of SFB_1 mRNA of *P. mume* (GenBank accession no. AB101440), derived from a functional *SFB* gene (YAMANE *et al.* 2003c), is only 11 bp from the stop codon of the relevant open reading frame. As a result, it does not seem likely that, for *SFB*, a short distance between the stop codon and the poly(A) tail leads to nonfunctionality.

The relative abundance of SFB_{36} mRNA with a poly(A) tail compared to total pollen mRNA was also examined. Total pollen mRNA of MSU 27e 9 (47) was separated into pollen mRNA with a poly(A) tail and without a poly(A) tail. A SFB_{36} -specific RT-PCR product

FIGURE 6.—RT-PCR for the S_{36} variants. (A) Style RT-PCR with PcS36ab-RNase-F and PcS36ab-RNase-R primers to detect expression of the S_{36} RNase variants. (B) Pollen RT-PCR with PcSFB36ab-F and PcSFB36-R primers to detect the expression of the SFB_{36} variants. Expression of the *actin* gene was used as a reference. Lane abbreviations are the following: C59, Cigány 59; Mon, Montmorency; P114, Pandý 114; RS, Rheinische Schattenmorelle; UF, Újfehértói fürtös. (C) RT-PCR with poly(A)⁺ and poly(A)⁻ pollen RNA for MSU 27e 9 (47) ($S_{13}'S_{36a}S_{36b}S_x$) (lanes 3 and 4, respectively) using the primers PcSFB36ab-F and PcSFB36ab-R. Expression of the *actin* and SFB_{13}' genes were used as references (lanes 1 and 2, respectively) with the primer pairs ActF1/ActR1 and PcSFB13-F/PcSFB13-R, respectively.

was not amplified using the poly(A)⁺ mRNA whereas SFB_{13}' -specific and *actin*-specific RT-PCR products were amplified from the poly(A)⁺ mRNA. In contrast, a SFB_{36} -specific RT-PCR product was amplified using poly(A)⁻ mRNA (Figure 6C). This suggests that the majority of SFB_{36} transcripts are not associated with a poly(A) tail. The three products amplified from the poly(A)⁺ mRNA that were of smaller than expected size (721 bp)—frA, frB, and frC—were sequenced and determined to have high identity (99%) with the *P. persica* Hakuto 18S ribosomal RNA (GenBank accession no. AY179530) (Figure S9). As a result, these small fragments were derived from nonspecific amplification.

Genetic behavior of the S_{36} haplotype variants: The functionality of the S haplotypes identified were tested using progeny populations derived from self-pollination. In these populations, each S haplotype tested should segregate in both the eggs and the pollen grains in an expected 1:1 ratio. Therefore, if an S haplotype is functional in the style and pollen, the pollen with that S haplotype would be incompatible, and the frequency of that S haplotype in the progeny should be 1/2, as in the eggs. However, if an S haplotype has lost either pollen or stylar function, the pollen carrying it would be compatible and it would be expected to be present in the

progeny in a 3:1 ratio. Using this strategy, we previously determined that the S_{36a} haplotype (S_a) in Montmorency, RS, and Surefire and the S_{36b} haplotype (S_d) in UF are nonfunctional (HAUCK *et al.* 2006b).

We further tested the functionality of the S_{36a} and S_{36b} haplotypes using self-pollinated progenies of Meteor and MSU III 18 (12) (Table 3). The S_{36a} and S_{36b} haplotypes in these two selections segregated in a 3:1 ratio, indicating that these S haplotypes are nonfunctional. In Meteor, only pollen containing the S_{13m} , S_{36a} , and/or S_{36b} would be capable of self-fertilization as its S_{33} has been shown to be functional (TSUKAMOTO *et al.* 2008). The S_{36b3} haplotype from MSU III 18 (12) was also determined to be nonfunctional as the segregation fits a 3:1 ratio, not a 1:1 one (Table 3). For this selection, only types containing S_{13}' , S_{36a} , and/or S_{36b3} would be capable of self-fertilization as S_1 was shown to be functional and segregates in a 1:1 ratio (data not presented). For Cigány 59, the S_9 and S_{26} haplotypes were previously shown to be functional (HAUCK *et al.* 2006b); therefore, the only SC pollen type would be $S_{6m2}S_{36b2}$ (Table 3). All the progeny resulted from this pollen type, indicating that the S_{36b2} haplotype is also nonfunctional.

Although all four S_{36} haplotype variants were determined in genetic tests to be nonfunctional, sequenc-

TABLE 3

Segregation of S_{36a} , S_{36b} , S_{36b2} , and S_{36b3} in self-pollinated progeny to determine the functionality of each S haplotype

S haplotype being tested	Parent (S genotype)	No. of progeny observed ^a	Segregation of S haplotypes		χ^2	P -value
			Observed ratio (present:absent)	Expected ratio ^b (present:absent)		
S_{36a}	Meteor ($S_{13m}S_{33}S_{36a}S_{36b}$)	83	64: 19	1:1	24.4	<0.0001
	MSU III 18 (12) ($S_1S_{13}'S_{36a}S_{36b3}$)	41	31: 10	3:1	0.197	0.6573
S_{36b}	Meteor ($S_{13m}S_{33}S_{36a}S_{36b}$)	83	63: 20	1:1	10.8	0.001
				3:1	0.008	0.9282
S_{36b2}	Cigány 59 ($S_{6m2}S_9S_{26}S_{36b2}$)	59	59: 0	1:1	22.3	<0.0001
				3:1	0.036	0.8492
S_{36b3}	MSU III 18 (12) ($S_1S_{13}'S_{36a}S_{36b3}$)	41	30: 11	1:0	—	—
				1:1	8.8	0.003
				3:1	0.073	0.7868

^a Complete progeny S -allele segregation data are presented in Table S3.^b A 1:1 ratio is expected if the S haplotype is fully functional, resulting in pollen rejection. A nonfunctional S haplotype would not result in pollen rejection; therefore, the S haplotype would be transmitted to the progeny at a frequency >50%.

ing conclusively revealed the molecular basis of this loss of function for only the S_{36b2} RNase (premature stop codon). In addition, the RT-PCR results suggested that the S -RNase and the SFB from the S_{36a} and S_{36b} haplotypes are transcribed. This left open the possibility that $S_{36a}S_{36b}$ (S_aS_d) pollen could be incompatible due to complementation between a functional S-RNase and a nonfunctional SFB or vice versa (HAUCK *et al.* 2006b). For example, if S_{36a} has a nonfunctional S-RNase and S_{36b} has a nonfunctional SFB, it is conceivable that $S_{36a}S_{36b}$ pollen would be incompatible in a $S_{36a}S_{36b}$ style due to the presence of a functional S_{36b} RNase and functional SFB_{36a}. To test this possibility, we reciprocally crossed UF and Montmorency. Among the 38 progeny of UF $\times$ Montmorency, the S_{36a} haplotype was present in 31 individuals (Table 4). This indicates that Montmorency pollen with the S_{36a} haplotype was compatible in the UF style containing the S_{36b} haplotype. In the reciprocal cross, the S_{36b} haplotype was present in 39 of the 60 progeny. Therefore, UF pollen with the S_{36b} haplotype was compatible in the Montmorency style containing the S_{36a} haplotype (Table 4). Taken together, these results disprove our previous hypothesis of com-

plementation between a functional S-RNase and a non-functional SFB.

Evidence for a lack of successful gametes with two S_{36} homologs: Genetic studies were conducted to test other hypotheses for why sour cherry individuals with two functional S haplotypes and two nonfunctional S_{36} haplotype variants were SI. We hypothesized that either (1) pollen grains and eggs containing two S_{36} haplotype variants are not formed during meiosis or (2) the presence of these two S haplotypes results in a lethal gamete. To explore these possibilities, we took advantage of the interfertility of sweet and sour cherry that resulted in triploid progeny, simplifying our ability to determine the gametes from the tetraploid sour cherry parent. Each sour cherry parent used had two S_{36} variants (either $S_{36a}S_{36b}$ or $S_{36a}S_{36b3}$) and two other S haplotypes ($S_{13m}S_{33}$, $S_{13}'S_{14}$, S_1S_4 , S_1S_{13}' , or S_4S_6 identified as $S_{x1}S_{x2}$ in Table 5 and Table 6). For each interspecific cross, there should be 12 or 6 progeny genotypes, depending on whether the S_{x1} or S_{x2} in the sour cherry parent matched none or one of the S haplotypes in the sweet cherry parent. For example, the sour cherry $S_4S_6S_{36a}S_{36b}$ should produce the following six

TABLE 4

Genetic behavior between S_{36a} and S_{36b} in reciprocal crosses

Parents (S genotype)	No. of progeny observed	Segregation ratio of pollen-derived S_{36} haplotype in the progeny		Expected ratio
		S_{36} haplotype	Present:absent	
UF ($S_1'S_4S_{35}S_{36b}$) $\times$ Montmorency ($S_6S_{13m}S_{35}S_{36a}$)	38	S_{36a}	31: 7	0:1 ^a
Montmorency ($S_6S_{13m}S_{35}S_{36a}$) $\times$ UF ($S_1'S_4S_{35}S_{36b}$)	60	S_{36b}	39:21	0:1

^a A 0:1 ratio is expected if S_{36a} RNase and SFB_{36b}, S_{36b} RNase and SFB_{36a} interact complementarily. Chi-square analysis is not possible when an expected class number is zero.

TABLE 5
Pollinations to test for the presence of $S_{36a}S_{36b}$ eggs in sour $\times$ sweet cherry crosses

Parents (S genotype): sour cherry ($S_{x1}S_{x2}S_{36a}S_{36b}$) ^a $\times$ sweet cherry	No. of progeny	S genotypes expected by one-allele-match model (actual no. of progeny segregated)			
		By egg cell of $S_{36a}S_{36b}$	By egg cell of $S_{x1}S_{36a}$ or $S_{x2}S_{36a}$	By egg cell of $S_{x1}S_{36b}$ or $S_{x2}S_{36b}$	By egg cell of $S_{x1}S_{x2}$
Meteor ($S_{13m}S_{33}S_{36a}S_{36b}$) $\times$ Hedel ^b (S_3S_5)	31	$S_3S_{36a}S_{36b}$ (0) $S_5S_{36a}S_{36b}$ (0)	$S_3S_{13m}S_{36a}$ (2) $S_5S_{13m}S_{36a}$ (4) $S_3S_{33}S_{36a}$ (1) $S_5S_{33}S_{36a}$ (3)	$S_3S_{13m}S_{36b}$ (4) $S_5S_{13m}S_{36b}$ (5) $S_3S_{33}S_{36b}$ (5) $S_5S_{33}S_{36b}$ (2)	$S_3S_{13m}S_{33}$ (0) $S_5S_{13m}S_{33}$ (5)
III 18 (12) ($S_1S_{13}'S_{36a}S_{36b3}$) $\times$ Hedel (S_3S_5)	26	$S_3S_{36a}S_{36b3}$ (0) $S_5S_{36a}S_{36b3}$ (0)	$S_1S_3S_{36a}$ (2) $S_1S_5S_{36a}$ (1) $S_3S_{13}'S_{36a}$ (2) $S_5S_{13}'S_{36a}$ (6)	$S_1S_3S_{36b3}$ (4) $S_1S_5S_{36b3}$ (4) $S_3S_{13}'S_{36b3}$ (6) $S_5S_{13}'S_{36b3}$ (1)	$S_1S_3S_{13}'$ (0) $S_1S_5S_{13}'$ (0)
27 12 (54) ($S_4S_6S_{36a}S_{36b}$) $\times$ Hedel (S_3S_5)	38	$S_3S_{36a}S_{36b}$ (0) $S_5S_{36a}S_{36b}$ (0)	$S_3S_4S_{36a}$ (2) $S_4S_5S_{36a}$ (3) $S_3S_6S_{36a}$ (4) $S_5S_6S_{36a}$ (3)	$S_3S_4S_{36b}$ (8) $S_4S_5S_{36b}$ (2) $S_3S_6S_{36b}$ (6) $S_5S_6S_{36b}$ (8)	$S_3S_4S_6$ (1) $S_4S_5S_6$ (1)
27 12 (54) ($S_4S_6S_{36a}S_{36b}$) $\times$ Gold (S_3S_6)	13	$S_3S_{36a}S_{36b}$ (1)	$S_3S_4S_{36a}$ (5) $S_3S_6S_{36a}$ (2)	$S_3S_4S_{36b}$ (4) $S_3S_6S_{36b}$ (1)	$S_3S_4S_6$ (0)
27 23 (16) ($S_4S_6S_{36a}S_{36b}$) $\times$ Hedel (S_3S_5)	27	$S_3S_{36a}S_{36b}$ (0) $S_5S_{36a}S_{36b}$ (0)	$S_3S_4S_{36a}$ (2) $S_4S_5S_{36a}$ (2) $S_3S_6S_{36a}$ (2) $S_5S_6S_{36a}$ (5)	$S_3S_4S_{36b}$ (3) $S_4S_5S_{36b}$ (2) $S_3S_6S_{36b}$ (3) $S_5S_6S_{36b}$ (4)	$S_3S_4S_6$ (1) $S_4S_5S_6$ (3)
27 23 (16) ($S_4S_6S_{36a}S_{36b}$) $\times$ Rainier (S_1S_4)	7	$S_1S_{36a}S_{36b}$ (0)	$S_1S_4S_{36a}$ (1) $S_1S_6S_{36a}$ (1)	$S_1S_4S_{36b}$ (2) $S_1S_6S_{36b}$ (2)	$S_1S_4S_6$ (1)
27 23 (16) ($S_4S_6S_{36a}S_{36b}$) $\times$ Gold (S_3S_6)	7	$S_3S_{36a}S_{36b}$ (1)	$S_3S_4S_{36a}$ (2) $S_3S_6S_{36a}$ (1)	$S_3S_4S_{36b}$ (3) $S_3S_6S_{36b}$ (0)	$S_3S_4S_6$ (0)
27 23 (42) ($S_4S_6S_{36a}S_{36b}$) $\times$ Hedel (S_3S_5)	28	$S_3S_{36a}S_{36b}$ (1) $S_5S_{36a}S_{36b}$ (0)	$S_3S_4S_{36a}$ (2) $S_4S_5S_{36a}$ (4) $S_3S_6S_{36a}$ (3) $S_5S_6S_{36a}$ (1)	$S_3S_4S_{36b}$ (6) $S_4S_5S_{36b}$ (5) $S_3S_6S_{36b}$ (3) $S_5S_6S_{36b}$ (2)	$S_3S_4S_6$ (0) $S_4S_5S_6$ (1)
27 23 (42) ($S_4S_6S_{36a}S_{36b}$) $\times$ Rainier (S_1S_4)	7	$S_1S_{36a}S_{36b}$ (2)	$S_1S_4S_{36a}$ (1) $S_1S_6S_{36a}$ (1)	$S_1S_4S_{36b}$ (1) $S_1S_6S_{36b}$ (1)	$S_1S_4S_6$ (1)
27 23 (42) ($S_4S_6S_{36a}S_{36b}$) $\times$ Gold (S_3S_6)	3	$S_3S_{36a}S_{36b}$ (0)	$S_3S_4S_{36a}$ (0) $S_3S_6S_{36a}$ (2)	$S_3S_4S_{36b}$ (0) $S_3S_6S_{36b}$ (1)	$S_3S_4S_6$ (0)
Total	187	5/187 (2.7%)	67/187 (35.8%)	101/187 (54.0%)	14/187 (7.5%)

^a S_{x1} and S_{x2} are S haplotypes other than S_{36a} , S_{36b} , and S_{36b3} .

^b Hedel, Hedelfingen.

gamete types: S_4S_6 , S_4S_{36a} , S_4S_{36b} , S_6S_{36a} , S_6S_{36b} , and $S_{36a}S_{36b}$. This would result in 12 progeny types if the sweet cherry parent did not have the S_4 or S_6 allele or in 6 progeny types if the sweet cherry parent had the S_4 or S_6 allele (in which case pollen grains containing the matching S allele would be incompatible). A total of 187 progeny were genotyped from 10 sour $\times$ sweet cherry crosses (Table 5). The vast majority of the progeny were formed from sour cherry eggs that contained only one S_{36} variant. Only 5 (2.7%) and 14 (7.5%) progeny resulted from eggs with two or zero S_{36} variants, respectively. The presence of these 5 progeny resulting from $S_{36a}S_{36b}$ eggs indicates that these eggs are not lethal.

A total of 101 progeny were S genotyped from 10 sweet $\times$ sour cherry crosses (Table 6). Only one progeny individual resulted from a sour cherry pollen grain with the S_{36a} and S_{36b} haplotypes, e.g., the progeny individual $S_3S_{36a}S_{36b}$ obtained from the cross between Hedelfin-

gen and MSU 27 23 (16). This indicates that $S_{36a}S_{36b}$ pollen is not lethal; instead, it is either rarely formed or uncompetitive compared with pollen of other S genotypes. This result is consistent with the genetic data from 86 and 41 progeny individuals from self-pollination of Meteor and MSU III 18 (12), respectively, where there was no evidence for progeny resulting from a pollen gamete with two S_{36} haplotypes (Table S3). This conclusion also provides an explanation for why the sour cherry cultivar Tarina ($S_1S_4S_{36a}S_{36b}$) is SI. In this case, the only SC pollen type, $S_{36a}S_{36b}$, rarely contributes to fruit and seed set (Figure 7).

Only 1 of the 101 progeny individuals from the sweet $\times$ sour cherry crosses resulted from a pollen grain that had no S_{36} variants (Table 6). This successful $S_{x1}S_{x2}$ sour cherry pollen was S_1S_{13}' . The lack of any progeny resulting from the $S_{x1}S_{x2}$ pollen types $S_{13}'S_{14}$, S_1S_4 , and S_4S_6 is unlikely to be solely due to the lethality or lack of

TABLE 6
Pollination to test for the presence of $S_{36a}S_{36b}$ pollen in sweet $\times$ sour cherry crosses

Parents (S genotype): sweet cherry $\times$ sour cherry ($S_{x1}S_{x2}S_{36a}S_{36b}$) ^a	No. of progeny	S genotypes expected by one-allele-match model (actual no. of progeny segregated)			
		By pollen of $S_{36a}S_{36b}$	By pollen of $S_{x1}S_{36a}$ or $S_{x2}S_{36a}$	By pollen of $S_{x1}S_{36b}$ or $S_{x2}S_{36b}$	By pollen of $S_{x1}S_{x2}$
Hedel ^b (S_3S_5) $\times$ ET ^c ($S_{13}'S_{14}S_{36a}S_{36b}$)	8	$S_3S_{36a}S_{36b}$ (0) $S_5S_{36a}S_{36b}$ (0)	$S_3S_{13}'S_{36a}$ (1) $S_5S_{13}'S_{36a}$ (1) $S_3S_{14}S_{36a}$ (1) $S_5S_{14}S_{36a}$ (2)	$S_3S_{13}'S_{36b}$ (2) $S_5S_{13}'S_{36b}$ (0) $S_3S_{14}S_{36b}$ (1) $S_5S_{14}S_{36b}$ (0)	$S_3S_{13}'S_{14}$ (0) $S_5S_{13}'S_{14}$ (0)
Summit (S_1S_2) $\times$ Tarina ($S_1S_4S_{36a}S_{36b}$)	3	$S_1S_{36a}S_{36b}$ (0) $S_2S_{36a}S_{36b}$ (0)	$S_1S_4S_{36a}$ (1) $S_2S_4S_{36a}$ (0)	$S_1S_4S_{36b}$ (0) $S_2S_4S_{36b}$ (2)	(0) ^e
EF ^d (S_3S_4) $\times$ Tarina ($S_1S_4S_{36a}S_{36b}$)	5	$S_3S_{36a}S_{36b}$ (0) $S_4S_{36a}S_{36b}$ (0)	$S_1S_3S_{36a}$ (0) $S_1S_4S_{36a}$ (1)	$S_1S_3S_{36b}$ (1) $S_1S_4S_{36b}$ (3)	(0) ^e
Hedel (S_3S_5) $\times$ Tarina ($S_1S_4S_{36a}S_{36b}$)	11	$S_3S_{36a}S_{36b}$ (0) $S_5S_{36a}S_{36b}$ (0)	$S_1S_3S_{36a}$ (2) $S_1S_5S_{36a}$ (0) $S_3S_4S_{36a}$ (3) $S_4S_5S_{36a}$ (0)	$S_1S_3S_{36b}$ (1) $S_1S_5S_{36b}$ (2) $S_3S_4S_{36b}$ (2) $S_4S_5S_{36b}$ (1)	$S_1S_3S_4$ (0) $S_1S_4S_5$ (0)
Gold (S_3S_6) $\times$ Tarina ($S_1S_4S_{36a}S_{36b}$)	14	$S_3S_{36a}S_{36b}$ (0) $S_6S_{36a}S_{36b}$ (0)	$S_1S_3S_{36a}$ (0) $S_1S_6S_{36a}$ (1) $S_3S_4S_{36a}$ (2) $S_4S_6S_{36a}$ (1)	$S_1S_3S_{36b}$ (4) $S_1S_6S_{36b}$ (3) $S_3S_4S_{36b}$ (1) $S_4S_6S_{36b}$ (2)	$S_1S_3S_4$ (0) $S_1S_4S_6$ (0)
Summit (S_1S_2) $\times$ III 18 (12) ($S_1S_{13}'S_{36a}S_{36b3}$)	6	$S_1S_{36a}S_{36b3}$ (0) $S_2S_{36a}S_{36b3}$ (0)	$S_1S_{13}'S_{36a}$ (0) $S_2S_{13}'S_{36a}$ (3)	$S_1S_{13}'S_{36b3}$ (2) $S_2S_{13}'S_{36b3}$ (1)	(0) ^e
Hedel (S_3S_5) $\times$ III 18 (12) ($S_1S_{13}'S_{36a}S_{36b3}$)	23	$S_3S_{36a}S_{36b3}$ (0) $S_5S_{36a}S_{36b3}$ (0)	$S_1S_3S_{36a}$ (2) $S_1S_5S_{36a}$ (3) $S_3S_{13}'S_{36a}$ (0) $S_5S_{13}'S_{36a}$ (3)	$S_1S_3S_{36b3}$ (3) $S_1S_5S_{36b3}$ (3) $S_3S_{13}'S_{36b3}$ (4) $S_5S_{13}'S_{36b3}$ (4)	$S_1S_3S_{13}'$ (1) $S_1S_5S_{13}'$ (0)
Schmidt (S_2S_4) $\times$ 27 12 (54) ($S_4S_6S_{36a}S_{36b}$)	1	$S_2S_{36a}S_{36b3}$ (0) $S_4S_{36a}S_{36b3}$ (0)	$S_2S_6S_{36a}$ (1) $S_4S_6S_{36a}$ (0)	$S_2S_6S_{36b}$ (0) $S_4S_6S_{36b}$ (0)	(0) ^e
Hedel (S_3S_5) $\times$ 27 12 (54) ($S_4S_6S_{36a}S_{36b}$)	16	$S_3S_{36a}S_{36b}$ (0) $S_5S_{36a}S_{36b}$ (0)	$S_3S_4S_{36a}$ (2) $S_4S_5S_{36a}$ (1) $S_3S_6S_{36a}$ (3) $S_5S_6S_{36a}$ (2)	$S_3S_4S_{36b}$ (0) $S_4S_5S_{36b}$ (1) $S_3S_6S_{36b}$ (4) $S_5S_6S_{36b}$ (3)	$S_3S_4S_6$ (0) $S_4S_5S_6$ (0)
Hedel (S_3S_5) $\times$ 27 23 (16) ($S_4S_6S_{36a}S_{36b}$)	14	$S_3S_{36a}S_{36b}$ (1) $S_5S_{36a}S_{36b}$ (0)	$S_3S_4S_{36a}$ (1) $S_4S_5S_{36a}$ (0) $S_3S_6S_{36a}$ (3) $S_5S_6S_{36a}$ (2)	$S_3S_4S_{36b}$ (0) $S_4S_5S_{36b}$ (3) $S_3S_6S_{36b}$ (4) $S_5S_6S_{36b}$ (0)	$S_3S_4S_6$ (0) $S_4S_5S_6$ (0)
Total	101	1/101 (1.0%)	42/101 (41.6%)	57/101 (56.4%)	1/101 (1.0%)

^a S_{x1} and S_{x2} are S haplotypes other than S_{36a} , S_{36b} and S_{36b3} .

^b Hedel, Hedelfingen.

^c ET, Englaise Timpurii.

^d EF, Emperor Francis.

^e Zero progeny were expected and none were obtained as $S_{x1}S_{x2}$ pollen grains are rejected by the functional S_{x1} and S_{x2} RNases in the style.

competitiveness of all these pollen types. Instead, the rarity of $S_{x1}S_{x2}$ gametes and gametes with two S_{36} variants most likely results from preferential meiotic pairing of the S_{36} variant chromosomes. Therefore, our data suggest that when two variants of the S_{36} haplotype are present in a sour cherry individual, the two chromosomes carrying the S_{36} haplotypes preferentially pair (Figure S10, A and B), resulting in only four gamete types (Figure S10C). However, when only one S_{36} variant is present in an individual, this S_{36} variant-containing chromosome can pair with any of the other three chromosomes (Figure S10, D–I), resulting in six gamete types (Figure S10J). For example, HAUCK *et al.* (2006b) reported that

RS ($S_6S_{13}'S_{26}S_{36a}$) could form six types of gametes: S_6S_{26} , $S_{13}'S_{36a}$, S_6S_{36a} , $S_{13}'S_{26}$, S_6S_{13}' , and $S_{26}S_{36a}$ (Figure S10J).

DISCUSSION

According to the one-allele-match model, three sour cherry individuals with the $S_4S_6S_{36a}S_{36b}$ genotype [MSU 27 12 (54), MSU 27 23 (16), and MSU 27 23 (42)] should be SC due to the compatibility of $S_{36a}S_{36b}$ pollen. Our previous finding that these individuals were SI provided an exception to this model. Using crosses, we demonstrated a very low transmission of $S_{36a}S_{36b}$ gametes to their self-pollinated offspring. Therefore, self-fertilization was

FIGURE 7.—Schematic diagram of self-pollination of the self-incompatible cultivar Tarina ($S_1S_4S_{36a}S_{36b}$). Tarina frequently forms four types of gametes: S_1S_{36a} , S_4S_{36b} , S_4S_{36a} , and S_1S_{36b} (open ovals), and rarely forms two types of gametes, S_1S_4 and $S_{36a}S_{36b}$ (shaded ovals). According to the one-allele match model, all types of pollen except for rarely generated self-compatible pollen with $S_{36a}S_{36b}$ are rejected by the functional S_1 and S_4 RNases in the style.

restricted, not by GSI or lethality, but by the very low frequency of transmission of the only pollen type that would be SC, a unique case where, despite the presence of two nonfunctional S haplotypes, the breakdown of SI in *Prunus* essentially does not occur. In this special case, the presence of two nonfunctional S_{36} variants is not enough for sour cherry to be SC, and another nonfunctional S haplotype is needed. Three SC sour cherry cultivars—Englaise Timpurii ($S_{13}'S_{14}S_{36a}S_{36b}$), Meteor ($S_{13m}S_{33}S_{36a}S_{36b}$), and MSU III 18 (12) ($S_1S_{13}'S_{36a}S_{36b3}$)—represent examples of this, as they each have a third nonfunctional S haplotype.

For all the previously studied S haplotype combinations and several isozyme loci (BEAVER and IEZZONI 1993; BOŠKOVIĆ *et al.* 2006; HAUCK *et al.* 2006b), gamete types resulting from homologous and less frequently homeologous pairing were observed. In contrast, the rare occurrence of gametes with two S_{36} variants is unique, suggesting preferential pairing of the chromosomes containing the S_{36} haplotypes. S_{36a} , S_{36b} , and S_{36b2} have been identified in the sour cherry progenitor *P. fruticosa* (T. TSUKAMOTO and A. F. IEZZONI, unpublished results), but not in the sweet cherry progenitor, suggesting that the S_{36} variants are derived from *P. fruticosa* and thus would be on chromosomes that would preferentially pair at meiosis. This is in contrast to the other S haplotypes in sour cherry (S_1 , S_4 , S_6 , S_9 , S_{12} , S_{13} , S_{14} , and S_{16}) that have all been identified in sweet cherry. Since the underlying mechanism limiting successful fertilization by gametes with two S_{36} haplotypes does not involve

GSI, our findings support the one-allele-match model for genetic control of SI and SC in sour cherry. The incompatibility of 2x pollen in *Prunus* distinguishes GSI in *Prunus* from that in the Solanaceae. Specifically, the incompatibility and compatibility of heteroallelic pollen in *Prunus* (sour cherry) and the Solanaceae, respectively, must be considered when developing functional models to explain the allele specificity of the incompatibility reaction.

Sequence analyses found no unambiguous primary defect leading to loss of function for the S_{36a} , S_{36b} , and S_{36b3} haplotypes. We were also unable to find a functional S_{36} haplotype in cherry for comparison. However, sequence analysis of the S_{36} variants provides some useful hints for future functional tests. Since all the S_{36} variants are nonfunctional, it is possible that the mutation(s) responsible for loss of function arose before the divergence of the different variants. If so, this mutation should be present in all the variants. A candidate could be the replacement of the amino acid residue at position 188 (from tyrosine to phenylalanine) of the SFB protein. This replacement is a change from a polar to a nonpolar residue. Furthermore, this tyrosine residue is conserved in all other known functional SFB proteins, suggesting that it might be important for function and that its mutation might cause malfunction of the protein. However, there is currently no biochemical or molecular evidence supporting the importance of this tyrosine residue in SFB function.

A second possible explanation for the nonfunctionality of the S_{36} variants is the presence of a non-autonomous *Helitron* element 38 bp downstream of the stop codon of *SFB* in all four S_{36} variants, which does not abolish the initiation of transcription. Eukaryotic genes usually are associated with imprecise termination of transcription; however, they require AATAAA [AAUAAA in mRNA, the poly(A) signal] for polyadenylation. This signal is not present in the 38 bp upstream of the *Helitron* element. However, it is present in the *Helitron* sequence, after a GC-rich stem loop and a region composed of consecutive T's, which are known to cause pausing of RNA polymerase and unwinding of RNA-DNA helices. Accordingly, some transcripts may not have the poly(A) signal. If few transcripts are polyadenylated, this may significantly affect the life span of the mRNA and the generation of the protein. Our results support this possibility in that the vast majority of S_{36} transcripts do not have a poly(A) tail, suggesting that the presence of the *Helitron* element interferes with the polyadenylation process. This may significantly affect the life span of the *SFB* mRNA and the generation of the protein. However, the real cause for the loss of function for the S_{36} variants awaits future biochemical analysis to test whether the absence of the poly(A) tail is responsible for the nonfunctional S_{36} haplotype. Interestingly, *Helitron* elements also seem to be involved in structural rearrangements of the Arabidopsis

Slocus (SHERMAN-BROYLES *et al.* 2007), suggesting that this element might have a widespread role in the evolution of self-compatibility.

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Molecular and Genetic Analyses of Four Nonfunctional *S* Haplotype Variants Derived from a Common Ancestral *S* Haplotype Identified in Sour Cherry (*Prunus cerasus* L.)

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FIGURE S1.—Amino acid sequence alignment of S_{36a} and S_{36b} RNases obtained from sour cherry and that of functional S-RNases from sweet and sour cherry. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. Conserved amino acids are shown on a darkened background. The five conserved regions, C1, C2, C3, RC4, and C5 (USHIJIMA *et al.* 1998), are marked with solid boxes, and the hypervariable region, RHV (USHIJIMA *et al.* 1998), reported in the rosaceous S-RNases is marked with a dotted box. S-RNases used for the alignment are eleven S-RNases from sweet cherry (*P. avium*) (PaS1-RNase: AB028153, PaS3-RNase: AB010306, PaS4-RNase: AB028154, PaS5-RNase: AJ298314, PaS6-RNase: AB010305, PaS7-RNase: EU035974, PaS12-RNase: AY259115, PaS13-RNase: DQ385842, PaS23-RNase: AY259114, PaS24-RNase: AY259112, PaS25-RNase) and four from sour cherry (*P. cerasus*) (PcS26-RNase: EU035975, PcS33-RNase: EU054325, PcS34-RNase: EU054326, and PcS35-RNase: EU054327).

PcSFB36a	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTSKSWSDLGSSSFVCTHLHNVTAHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PcSFB36b	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTSKSWSDLGSSSFVCTHLHNVTAHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB1	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB2	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB3	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB4	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB5	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB6	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB7	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB9	1	MTFRLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB10	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB12	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	88
PaSFB13	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PaSFB16	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PcSFB1	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PcSFB26	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PcSFB33	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	90
PcSFB34	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
PcSFB35	1	MAFTLRKKE	ILIDILVRLPAKSLVRLFLCTCKSWDLIGSSSFVSTHLHNVTKHAHVYLLCLHHPNFEWAVDDDDPYVKOELOWSLFSN	89
F-box				
PcSFB36a	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSARKFRTPPMSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PcSFB36b	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSARKFRTPPMSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB1	90	QTEFEVYKLSHPLGNTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB2	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB3	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB4	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB5	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB6	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB7	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB9	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB10	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB12	89	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	178	
PaSFB13	90	QTEFEVYKLSHPLGNTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PaSFB16	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PcSFB1	90	QTEFEVYKLSHPLGNTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PcSFB26	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PcSFB33	91	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	180	
PcSFB34	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	179	
PcSFB35	90	ETFEKCFEHRHPIGSTEHYGIYSSNGLVCISDEILNFDSPHIHWNPSVRKRTPTPTSTNINIKFSYVALQFGFHPVNDYKAVRMMRTN	178	
V1				
PcSFB36a	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PcSFB36b	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB1	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB2	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB3	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB4	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB5	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB6	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB7	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB9	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB10	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB12	179	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	268	
PaSFB13	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PaSFB16	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PcSFB1	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PcSFB26	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PcSFB33	181	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	270	
PcSFB34	180	KDALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	269	
PcSFB35	179	ENALAVEVYSLRTDSWKMIETAPPWLKCTWOHMGTSFNGVAYHLLRGKPIFSIMSFDSGSEEFEEFIAPDAICSSWGLCIDVYKEQICL	268	
Vn				
PcSFB36a	270	LFREFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	357	
PcSFB36b	270	LFREFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	357	
PaSFB1	270	LDLFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PaSFB2	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	359	
PaSFB3	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PaSFB4	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	357	
PaSFB5	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	359	
PaSFB6	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	357	
PaSFB7	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PaSFB9	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PaSFB10	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PaSFB12	269	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	356	
PaSFB13	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PaSFB16	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	357	
PcSFB1	270	LDLFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PcSFB26	270	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	357	
PcSFB33	271	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PcSFB34	270	LDLFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	358	
PcSFB35	269	LSGFYCEEEMGKVDLWVLOEKRWKQCPFIYPS--CYRTMGISIDNELLQRRDLKKGVAIYLCNYESKQVLETGIGIKLAIRYGEI	355	
V2				
PcSFB36a	358	EFLFSITYIESVLLNDY	375	
PcSFB36b	358	EFLFSITYIESVLLNDY	375	
PaSFB1	359	EFLFAITYIESVLLNNH	376	
PaSFB2	360	EFLFAITYIESVLLNDY	377	
PaSFB3	359	EFLFAITYIESVLLNNH	376	
PaSFB4	358	EFLFAITYIESVLLNNH	375	
PaSFB5	360	EFLFAITYIESVLLNNH	376	
PaSFB6	358	EFLFAITYIESVLLNNH	375	
PaSFB7	359	EFLFSITYIESVLLNDY	374	
PaSFB9	359	EFLFSITYIESVLLNNH	376	
PaSFB10	359	EFLFSITYIESVLLNNH	376	
PaSFB12	357	EFLFAITYIESVLLNNH	376	
PaSFB13	359	EFLFAITYIESVLLNNH	376	
PaSFB16	358	EFLFAITYIESVLLNNH	376	
PcSFB1	359	EFLFAITYIESVLLNNH	376	
PcSFB26	358	EFLFSITYIESVLLNNH	375	
PcSFB33	359	EFLFAITYIESVLLNNH	376	
PcSFB34	359	EFLFAITYIESVLLNNH	376	
PcSFB35	356	EFLFAITYIESVLLNNH	371	

FIGURE S2.—Amino acid sequence alignment of SFB36a and SFB36b obtained from sour cherry and that of functional SFBs from sweet and sour cherry. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. Conserved amino acids are shown on a darkened background. The locations of the F-box motif, V1, V2, HVa and Hvb (IKEDA *et al.* 2004a), and Vn (NUNES *et al.* 2006) are indicated by solid boxes. The asterisks indicate the 3 amino acid residues that are conserved in all functional cherry SFBs but not in SFB36a and SFB36b. SFBs used for the alignment are twelve SFBs from sweet cherry (*P. avium*) (PaSFB1: AY805048, PaSFB2: AB111519, PaSFB3: AB096857, PaSFB4: AB111521, PaSFB5: AB111520, PaSFB6: AB096858, PaSFB7: EU035976, PaSFB9: DQ422809, PaSFB10: AY805053, PaSFB12: AY805054, PaSFB13: DQ385844, PaSFB16: AY805056) and five from sour cherry (*P. cerasus*) (PcSFB1: DQ827715, PcSFB26: EU035977, PcSFB33: EU054328, PcSFB34: EU054329, and PcSFB35: EU054330).

PcSFB36a	180	KDAFAVEFFSLGTD	SWKMIEAIPWLKCTWQHMQSIFSN	GVAHLLRKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKEHIC-L	269
PcSFB36b	180	KDAFAVEVFLGTD	SWKMIEAIPWLKCTWQHMQSIFSN	GVAHLLRKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKEHIC-L	269
PaSFB1	180	KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WRSC-IEVYKEQIC-L	269
PaSFB2	180	KNTLAVEVSLRRD	RWKMEIAPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICNL-WGLC-IDVYKEQIC-L	269
PaSFB3	180	KGALAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-REL-IDVYKEHIC-L	269
PaSFB4	180	KDALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLF-IDVYKEQIC-L	269
PaSFB5	180	KDALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYNDQIC-L	269
PaSFB6	180	KNALAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-ELC-IDVYKERV-L	269
PaSFB7	180	KNAFTVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFNPGSEEF	QEFIIAPDAICAP-WGLC-IDVYKGQIC-L	269
PaSFB9	180	KNALVVEVSLRTD	SWKMIEAIPWLKCTWQPFKGT	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WELC-IDVYKEQIC-L	269
PaSFB10	180	KNSFAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKEQIC-L	269
PaSFB12	179	KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRP-FALC-IDVYKEQIC-L	268
PaSFB13	180	KNTLAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKEQIC-L	269
PaSFB16	180	KNALVVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-ELC-IDVYKEQIC-L	269
PcSFB1	180	KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WRSC-IEVYKEQIC-L	269
PcSFB26	180	KNAFAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICTS-WGLF-IDVYNDQIC-L	269
PcSFB33	181	KGALAVEVSLRKD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICHP-WGLC-IDVYKEQIC-L	270
PcSFB34	180	KGALAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSANEFEF	IAPDDICSL-WGLC-IDVYKERIC-L	269
PcSFB35	179	ENAFAVEVSLSTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSSEKFEF	IAPDAICRS-WGLC-IDVYKEQIC-L	268
PaSFB1	180	KNVLAIVEVSLSTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRS-LGLC-IDVYKEHIC-L	269
PaSFB2	180	KNSLAIVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKENIC-L	269
PaSFB4	179	KNALAIVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRPS-EVC-IDVYKEQIC-L	268
PaSFB13		KNALAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHLLKGPISIMSFDSGSEEEF		
PaSFB14	180	KSALAVEVSLKRD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEKFEF	IAPDAICSL-WGLY-IDVYKEQIC-L	269
PaSFBc	180	KSALAVEVSLKRD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEKFEF	IAPDAICSL-WGLY-IDVYKEQIC-L	269
PcSfSFB3		KSALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRP-FALC-IDVYKEQIC-L	
PcSfSFB9		KNPLAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICNP-CELVIRIDVYKEQIC-L	
PcSfSFB10		KNALVVEVSLRAD	SWMMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	MAPDAICSS-WRSC-IDVYKEQIC-L	
PdSFB3		KGALAEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRS-LGLG-IDVYKEQIC-L	
PdSFB8		QNALAVEVSLRTD	SWTMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSL-WRCL-IDVYKEQIC-L	
PdSFB10		KGALAVEVSLKTY	CKWMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDDICSS-WGLC-IDVYKERIC-L	
PdSFB11	180	KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WRSC-IEVYKEQIC-L	269
PdSFB12		KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRP-FALC-IDVYKEQIC-L	
PdSFB13		KNPLAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICNP-CELVIRIDVYKEQIC-L	
PdSFB23		KGAVAEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRS-WGLC-IDVYKGQIC-L	
PdSFBa	179	KDAFAVEVSLSTD	SWKRIEIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRS-RGLF-IDVYKEQIC-L	268
PdSFBb	181	KSALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSL-WGL-IDVYKENIC-L	270
PdSFBc	179	KDFAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRS-RGLC-IDVYKEQIC-L	268
PdSFBd	180	QNALAVEVSLRTD	SWTMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSL-WRCL-IDVYKEQIC-L	269
PdSFBf		KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDDICTS-WGLC-IDVYKEQIC-L	
PdSFBk	180	KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRP-FALC-IDVYKEQIC-L	269
PdSFB5		KGALAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDDICSS-WGLC-IDVYKERIC-L	
PdSFB6		KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICGT-FGLC-IDVYKEQIC-L	
PdSFB9		KDPLVVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDVICSS-WGLC-IDVYKEQIC-L	
PmSFB1	180	KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-LGLC-IDVYKEQIC-L	269
PmSFB7	180	KNALAVEVSLRTD	FWKMIETIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICONS-WGLC-IDVYKEQIC-L	269
PmSFB9	180	KNALAEVSLGTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAISTP-WGLC-IDVYKEQIC-L	269
PpcSFB1	180	KGAVAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLY-IDVYKEQIC-L	269
PpcSFB5	180	KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSNPS-DLC-IDVYKEQIC-L	269
PpcSFB7	180	KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICONS-WGLC-IDVYKEQIC-L	269
PpcSFBa	180	KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF			215
PpcSFBb	180	KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF			215
PpcSFBc	179	KDFAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICONS-RGLC-IDVYKEQIC-L	268
PpSFB7		KGAFVVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICDT-WGLC-IDVYKEQIC-L	
PpSFB10	180	KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRPS-ELC-IDVYKEQIC-L	269
PpSFBa	180	KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICGT-FGLC-IDVYKEQIC-L	269
PpSFBb	180	KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICTS-WGLC-IDVYNDQIC-L	269
PpSFBc	180	KDAFTVEVSLGTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKEQIC-L	269
PpSFBd		KNPLAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICNP-CELVIRIDVYKEQIC-L	
PpSFBf	180	KDAFAVEVSLQTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSPS-DLC-IDVYKEQIC-L	269
PpSFBf		KDAFAVEVSLGTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICNP-CTLC-IDVYKEQIC-L	
PpSFBg		KNALVVEVSLRAD	SWMMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WRSC-IDVYKEQIC-L	
PpSFBh	179	KDFAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSL-WGLC-IDVYKEQIC-L	268
PpSFBk		KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WKLC-IDVYKEQIC-L	
PpSFB1		KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRPS-ELC-IDVYKERIC-L	
PpSFB3-1		KNALAVEVSLRTD	SWKIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICGT-WGLC-IDVYKEQIC-L	
PpSFB3-2		KNALAVEVSLRTD	SWKIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICGT-WGLC-IDVYKEQIC-L	
PpSFB4		KNAFAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKEQIC-L	
PpSFB5		KGALAVEVSLRAD	SWKILEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICNP-CALC-IDVYKEQIC-L	
PpSFB7-1		KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAISGT-FGLC-IDVYKEQIC-L	
PpSFB7-2		KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAISGT-FGLC-IDVYKEQIC-L	
PpSFB8		KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDSCSS-ELC-IDVYKEQIC-L	
PpSFB9		KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRPS-ELC-IDVYKERIC-L	
PpSFB10		KSAMVQVSLRTD	TWKMIETIPWLKCTWQHMKGTF	ICNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICTPS-DLC-IDVYKEQIC-L	
PpSFB12		KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKEQIC-L	
PpSFB13		KTAFTVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICAP-WGLC-IDVYKEQIC-L	
PpSFB14		KNALAVEVSLKTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDPGTEEF	KFEIAPDAICSP-WELC-IDVYKEQIC-L	
PpSFB15		KNALAVEVSLRAD	SWKIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICNL-WGLC-IDVYKEQIC-L	
PpSFB16		KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICNP-CTLC-IDVYKEQIC-L	
PpSFB17		KGALAVEVSLRKD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICGT-WGLC-IDVYKEQIC-L	
PpSFB18		KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRS-LGLC-IDVYKEQIC-L	
PpSFB19		KNALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLC-IDVYKEQIC-L	
PpSFB20		KGAVAEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICAS-WGLC-IDVYKEQIC-L	
PpSFB21		KSALAVEVSLRAD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WGLG-IDVYKEQIC-L	
PpSFB22		KGAVAEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICRP-MELC-IDVYKEQIC-L	
PtSFB8	180	KGALAVEVSLRTD	SWKMIEAIPWLKCTWQHMKGTF	FNGVAYHIEKGPIFSIMSFDSGSEEEF	IAPDAICSS-WRSC-IDVYKEQIC-L	269

*

Vn

FIGURE S3.—Amino acid sequence alignment between positions 180 and 269 of SFB36a and SFB36b and that of 85 functional SFBs obtained from cherry and other *Prunus* species. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. The conserved tyrosine at position 188 in all functional *Prunus* SFBs but not SFB36a and SFB36b, is shown on a darkened background and identified by a dagger symbol. The location of the variable region Vn (NUNES *et al.* 2006) is indicated by a solid box.

FIGURE S3.— (continued) Functional *Prunus* SFBs used for the alignment are twelve SFBs from *P. avium* (see supplemental Figure 2), five from *P. cerasus* (see supplemental Figure 2), six from *P. armeniaca* (ParSFB1: AY587563, ParSFB2: AY587562, ParSFB4: AY587565, ParSFB13: EF062342, ParSFB14: DQ897929, ParSFBc: DQ422946), three from *P. cerasifera* (PcsfSFB3: AM746952, PcsfSFB9: AM746953, PcsfSFB10: AM746954), fourteen from *P. dulcis* (PdSFB3: EU293149, PdSFB8: EU293148, PdSFB10: AM746958, PdSFB11: EF061758, PdSFB12: AM746959, PdSFB13: AM746960, PdSFB23: EU310403, PdSFB30: AM711127, PdSFBa: AB092966, PdSFBb: AB092967, PdSFBc: AB079776, PdSFBd: AB081648, PdSFBf: EU310402, PdSFBk: AB252408), three from *P. domestica* (PdoSFB5: AM746955, PdoSFB6: AM746956, PdoSFB9: AM746957), three from *P. mume* (PmSFB1: AB101440, PmSFB7: AB101441, PmSFB9: AB092645), six from *P. pseudocerasus* (PpcSFB1: EU253963, PpcSFB5: EU253964, PpcSFB7: EU253965, PpcSFBa: EU267941, PpcSFBb: EU267942, PpcSFBc: EU267943), eleven from *P. salicina* (PsSFB7: DQ849085, PsSFB10: DQ849090, PsSFBa: AB252410, PsSFBb: AB252412, PsSFBc: DQ849084, PsSFBd: AM746962, PsSFBe: AB280794, PsSFBf: DQ849089, PsSFBg: AM746963, PsSFBh: DQ849118, PsSFBk: DQ992485), twenty one from *P. spinosa* (PspSFB1: DQ677607, PspSFB3-1: DQ677616, PspSFB3-2: DQ677615, PspSFB4: DQ677605, PspSFB5: DQ677600, PspSFB7-1: DQ677595, PspSFB7-2: DQ677592, PspSFB8: DQ677587, PspSFB9: DQ677588, PspSFB10: DQ677589, PspSFB12: DQ677598, PspSFB13: DQ677591, PspSFB14: DQ677621, PspSFB15: DQ677609, PspSFB16: DQ677601, PspSFB17: DQ677597, PspSFB18: DQ677620, PspSFB19: DQ677612, PspSFB20: DQ677611, PspSFB21: DQ677596, PspSFB22: DQ677610) and one from *P. tenella* (PtSFB8: DQ983369).

FIGURE S4.—Alignment of fosmid clones used to obtain nucleotide sequence of S_{36} haplotypes.

FIGURE S5.— Nucleotide alignments of upstream region of the *S-RNase* of the four *S*₃₆ haplotypes. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. Adenine of the start codon was positioned to be +1. Conserved nucleotides are shown on a darkened background. The positions and directions of the

FIGURE S6.— Nucleotide alignments of downstream region of the *S-RNase* of the four *S*₃₆ haplotypes. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. Adenine of the start codon was positioned to be +1. Conserved nucleotides are shown on a darkened background. The positions and directions of the primers used

FIGURE S7.—PCR digestion products for *SFB*₃₆ variant clones from ‘MSU 27e 9 (47)’ (*S*₁₃’*S*_{36a}*S*_{36b}*S*_x) and ‘Tamaris’ (*S*₁₃’*S*₁₆*S*₃₄*S*_{36b2}) obtained from 3’ RACE of pollen RNA. Colony PCR products amplified with CAPS markers PcSFB36ab-F and PcSFB36ab-R were digested with *Rsa*I. The PCR products of *SFB*_{36a} clones were digested into three fragments of 326 bp, 262 bp and 133 bp, whereas those of *SFB*_{36b} clones were digested into three fragments of 326 bp, 284 bp and 111 bp.

FIGURE S8.—Nucleotide alignments of *SFB*₃₆ variant clones obtained from 3' RACE of pollen RNA of 'MSU 27e 9 (47)' and 'Tamaris'. 27e 9 (47)-8, 27e 9 (47)-3 and Tamaris-1 encode *SFB*_{36a}, *SFB*_{36b} and *SFB*_{36b}, respectively. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. Adenine of the start codon was positioned to be +1. Conserved nucleotides are shown on a darkened background. The asterisks

FIGURE S9.— Nucleotide sequences of three products amplified in RT-PCR of poly (A)+ pollen RNA of ‘MSU 27e 9 (47)’ were aligned with nucleotide sequence of *Prunus persica* ‘Hakuto’ 18S ribosomal RNA (GenBank accession no: AY179530). The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. Conserved nucleotides are shown on a darkened background. The primer sequences used in RT-PCR are italicized and their positions are indicated by arrows.

FIGURE S10.—Chromosome pairing. Chromosome pairing of 'Tarina' ($S_1S_4S_{36a}S_{36b}$) (A and B); and generated gamete types from its meiosis (C). Chromosome pairing of 'Rheinische Schattenmorelle' ($S_6S_{13'}S_{26}S_{36a}$) (D-I); and generated gamete types from its meiosis (J). The S_{36} haplotypes are indicated in red and the other S haplotypes are indicated in blue.

TABLE S1

Primers designed to sequence the flanking regions of the *S-RNase* and the*SFB* for the *S₃₆* variants

Primer name	Primer sequence 5' → 3'
pCC1FOS-F	GGATGTGCTGCAAGGCGATTAAGTTGG
pCC1FOS-R	CTCGTATGTTGTGTGGAATTGTGAGC
T7	TAATACGACTCACTATAGGG
B4upF1	GAGCTTGAAATAAGGTTTG
B4upF2	AAACTAGATGGTGTAGCTC
B4upF3	CTGAATAGCATAAAAAGAAA
B4upR1	TTTCTTTTATGCTATTCAG
B4upF4	CTAATTTTAAATCGGTTTG
B4upR2	CAAACCGATTAAAAATTAG
B4upF5	GGCTACTTTGTTCTAAAAAG
B4upR3	ATGCTAACTTAATGAGATTTT
B4upR4	ATGTGAGCCACTTAACTT
PcS36ab-1945R	ACCTTATTTAAGCGAGTCGT
PcS36ab-1887R	CATTGGGCATAATTACCTC
PcS36ab-1712R	AGAAAATTGCTAGATTATTG
PcS36ab-1588R	TGTATTGCATTATTTTGTGTA
PcS36ab-1398F	GCACAACAAATGTATTTAAT
PcS36ab-1165F	TTCTTCTATATTCCTCTCATC
PcS36ab-1061R	GAGTTAAGAGTAAATCTTGCT
PcS36ab-1001R	TACTCGGTTCTAGTTTCTAC
PcS36ab-866R	GTTTGGTGTGATTTCAGTAT
PcS36ab-837R	AAGGTTAGTACGCATTAGG
PcS36ab-245R	TAGAAAAGGTCTTGAGTCAT
PcS36ab-214R	CTAATATAGTGGTTTGGAGC
PcS36ab322F	TAGATACCATTTCTTCTTAC
PcS36ab366F	TATGACTATTTTCAATTTGTG
PcS36ab1606F	ATGAATAATTGCACCTTACAG
PcS36ab1681F	TCTATATGTCATTTTACATGTA
PcS36ab2412F	ATCAATAATATAGACAATGCA
PcS36ab2502F	TCATTAAGTCAATCATTG
PcS36a-sp3283F	ATTTCTCTTCTGACTAACG
PcS36a-sp3326F	TTTGTTTAGTGTGCTAATG
PcS36b-sp3173F	CCAGAAATAAACCTGAATA
PcS36b-sp3322F	GTTTCCTTCAACTAAAAAG
PcSFB36ab-693F	CAGCATTATGTCATTCGATTCA
PcSFB36ab-845F	AACAAGTTGACTTATGGGTCCT

TABLE S2

PCR primer pairs designed to amplify the end sequences of the fosmid clones^a

Fosmid name (end name)	Primer sequence	Product
Primer name	5' → 3'	size (bp)
B4 (FOS-Forward end)		
B4-fosF-F	GCCTATGTGGTTAATGAGCTTC	489
B4-fosF-R	TAGTTTTCATCCAAAAATCTG	
B4 (FOS-Reverse end)		
B4-fosR-F	AACAGGCCTTAGTTGACACTTC	486
B4-fosR-R	GTCATATCCTGGAAAGAGGAAG	
Met8 (FOS-Forward end)		
Met8-fosF-F	CAAAAATCCTAACCCACAATG	446
Met8-fosF-R	GGACGTTCTGTGAGTCAC'TTAG	
C19 (FOS-Reverse end)		
C19-fosR-F	GCACGATTAATGTCGTATGTG	505
C19-fosR-R	CTGGTGTCTGATAACGAAGTATC	
III 18 (12) 3 (FOS-Forward end)		
III 18 (12) 3-fosF-F	CAATTCTAATTTGCTCAAACCC	594
III 18 (12) 3-fosF-R	TTTTTCCTTGATCAGCTCTACC	
III 18 (12) 3 (FOS-Reverse end)		
III 18 (12) 3-fosR-F	ATATTGTGGAATGAGTTCGTTG	306
III 18 (12) 3-fosR-R	CGCAACTAAGCTGTTCAAATAC	
III 18 (12) 7 (FOS-Forward end)		
III 18 (12) 7-fosF-F	TATCCAAGAGGTTCTTACGTG	640
III 18 (12) 7-fosF-R	ATATCTCGCTGTAACCTTCTCGC	
III 18 (12) 7 (FOS-Reverse end)		
III 18 (12) 7-fosR-F	GTATTTGAACAGCTTAGTTGCG	556
III 18 (12) 7-fosR-R	CCTCAAGGTCTGTATGGTGTTAG	
III 18 (12) 12 (FOS-Forward end)		
III 18 (12) 12-fosF-F	AATCCTAGCATCAGGATCAGAC	476
III 18 (12) 12-fosF-R	CCTAATATTGCAGGCGTCTTAC	
III 18 (12) 12 (FOS-Reverse end)		
III 18 (12) 12-fosR-F	GATGGAAGATGCATT'TAGTGAG	558
III 18 (12) 12-fosR-R	AGTTTGAACCCCTATGACAATG	

^a Annealing temperature is 65 °C, and extension time is 60 seconds for all primer pairs.

TABLE S3

Possible progeny *S* allele phenotypes from self-pollination of three sour cherry cultivars and the number of progeny individuals obtained

Parent (<i>S</i> genotype)	Possible <i>S</i> allele phenotypes	Number of progeny obtained
'Meteor' ($S_{13m}S_{33}S_{36a}S_{36b}$)	$S_{13m}S_{33}S_{36a}S_{36b}$	20
	$S_{13m}S_{36a}S_{36b}$	24
	$S_{13m}S_{33}S_{36a}$	8
	$S_{13m}S_{33}S_{36b}$	15
	$S_{33}S_{36a}S_{36b}$	0 ^a
	$S_{13m}S_{36a}$	12
	$S_{13m}S_{36b}$	4
	$S_{37a}S_{36b}$	0 ^a
	Total	83
'MSU III 18 (12)' ($S_I S_{13} S_{36a} S_{36b3}$)	$S_I S_{13} S_{36a} S_{36b3}$	16
	$S_{13} S_{36a} S_{36b3}$	5
	$S_I S_{13} S_{36a}$	1
	$S_I S_{13} S_{36b3}$	6
	$S_I S_{36a} S_{36b3}$	0 ^b
	$S_{13} S_{36a}$	9
	$S_{13} S_{36b3}$	2
	$S_{36a} S_{36b3}$	0 ^b
	$S_I S_{36b3}$	1 ^c
	$S_I S_{13}$	1 ^c
	Total	41
'Cigány 59' ($S_{6m2}S_9S_{26}S_{36b2}$)	$S_{6m2}S_9S_{26}S_{36b2}$	13
	$S_{6m2}S_9S_{36b2}$	11
	$S_{6m2}S_{26}S_{36b2}$	23
	$S_{6m2}S_{36b2}$	12
	Total	59

The possible *S* allele phenotypes take into consideration the self-incompatibility of pollen carrying the S_{33} haplotype for 'Meteor', S_I haplotype for 'MSU III 18 (12)', and S_9 and/or S_{26} haplotype for 'Cigány 59'.

^a This progeny class would only result from pollination with pollen of the genotype $S_{36a}S_{36b}$.

^b This progeny class would only result from pollination with pollen of the genotype $S_{36a}S_{36b3}$.

^c These progeny individuals would have been formed with pollen gametes produced by double reduction, e.g. $S_{36b3}S_{36b3}$ and $S_{13}S_{13}$, respectively. Double reduction has been previously documented in sour cherry for alleles at the *S*-locus (HAUCK *et al.* 2006b (Supplemental Figure S1); BOŠKOVIĆ *et al.* 2006).