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Nathanael R. Hauck

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Amy Iezzoni
Major Professor's Signature

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THE GENETIC CONTROL OF SELF-INCOMPATIBILITY IN SWEET AND SOUR CHERRY

By

Nathanael R. Hauck

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ABSTRACT

THE GENETIC CONTROL OF SELF-INCOMPATIBILITY
IN SWEET AND SOUR CHERRY

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Nathanael R. Hauck

Gametophytic self-incompatibility (GSI) is a common mechanism for preventing inbreeding in flowering plants. Typically, GSI in diploid species breaks down due to polyploidy resulting in self-compatible (SC) tetraploid species. The diploid sweet cherry and the tetraploid sour cherry represent an exception, as sour cherry individuals can be either self-incompatible (SI) or SC. SI is undesirable for cultivation due to the inefficiencies of growing pollinator varieties and the reliance on bees to ensure adequate cross-pollination. Therefore, sour cherry breeders should develop SC selections. Without an understanding of the genetic control of SI and SC in sour cherry, breeders are not able to predict the SI or SC phenotype of seedlings prior to the production of flowers, which typically occurs 3-5 years after planting. The availability of molecular markers to predict the SI or SC phenotype of a seedling could save valuable field space and evaluation time. The goal of this dissertation was to determine the genetic control of SI and SC in sour cherry. To do this, it was first necessary to determine which *S*-haplotypes exist in sweet cherry, one of the progenitors of sour cherry. RFLP analyses were used to determine the banding profiles for 14 sweet cherry *S*-haplotypes. Sour cherry was then found to contain six of these sweet cherry *S*-haplotypes (S_1, S_4, S_6, S_9, S_{12} and S_{13}) in addition to six unique *S*-haplotypes (S_{6c}, S_{6m}, S_a, S_b, S_d and S_e). Using inter-specific crosses between sweet and sour cherry and self-pollinations of sour cherry, four of the six

shared *S*-haplotypes (S_1, S_4, S_6, and S_9) and one of the unique *S*-haplotypes (S_b) were shown to be functional and capable of accomplishing *S*-haplotype-specific rejection of pollen. The other *S*-haplotypes in sour cherry (S_{13}, S_{6c}, S_{6m}, S_a, S_d, and S_e) were shown to be non-functional and incapable of initiating *S*-haplotype-specific rejection of pollen. Finally, a hypothesis regarding the genetic control of SI and SC in sour cherry was developed through the analysis of *S*-haplotype segregation in 794 progeny from six sour cherry self-populations and 15 inter-specific crosses between sweet and sour cherry. SI and SC predictions were verified using additional self-pollination and crossing experiments. The data suggests that the partial breakdown of SI in sour cherry is due to the accumulation of non-functional *S*-haplotypes that are incapable of *S*-haplotype-specific rejection of pollen, rather than due to the competition between pollen-*S* products in heteroallelic pollen, which is commonly observed in the Solanaceae. The implications of these findings on sour cherry breeding and on our knowledge of GSI are discussed.

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Introduction

Gametophytic self-incompatibility (SI) is a genetic mechanism that promotes out-crossing in many flowering plants (de Nettancourt 1977). SI is controlled by a single multi-allelic locus, called the *S*-locus, which is hypothesized to contain at least two genes involved in determining the specificity of the SI interaction between the pollen tubes and styles. The growth of pollen tubes is arrested within the style if the pollen and style contain the same specificity alleles.

Sour cherry (*Prunus cerasus* L.) is an allotetraploid species produced by the hybridization of the diploid sweet cherry (*P. avium* L.) and the tetraploid ground cherry (*P. fruticosa* Pall.) (Olden and Nybom, 1968). Whereas sweet cherry has a classic gametophytic SI system, sour cherry segregates for SI and self-compatibility (SC) (Lech and Tylus, 1983; Redalen 1984). The presence of SI sour cherry selections is unusual, since SI typically breaks down as a result of polyploidy (Livermore and Johnstone 1940; Crane and Lewis 1942; Stout and Chandler 1942; Brewbaker 1954; Pandey 1968). The reason for the partial breakdown of SI in sour cherry is unknown. In the Solanaceae, which has a similar gametophytic SI system, the breakdown of SI is apparently caused by the competition of pollen-*S* products in heteroallelic pollen, i.e. pollen containing two different pollen-*S* products.

For growers, SI sour cherry trees require mixed plantings with pollinator cultivars in their orchard and the use of bees to ensure proper cross pollination and fruit set. The need to use valuable orchard space for pollinators and the reliance on bees for adequate fruit set compel growers to desire SC cultivars rather than SI ones. Thus, sour cherry breeders are interested in breeding SC trees. Without markers for SC, the breeder may

have to wait three to five years after making a cross to determine if the tree is SI or SC. Therefore, the development of molecular markers for SC would facilitate sour cherry breeding and reduce the number of seedlings that a breeder would need to keep. However, without knowledge of the genetic control of SI and SC in sour cherry, it is impossible to develop molecular markers that could be used to screen for SC seedlings.

The ultimate goal of this research was to determine the genetic control of SI and SC in sour cherry to facilitate marker development. To reach this goal, it was first necessary to test the hypothesis that gametophytic SI in sour cherry involves the stylar *S*-RNase as the style specificity component. This involved the determination of the *S*-haplotypes in sour cherry and the relationship of these *S*-haplotypes to those in sweet cherry. With these *S*-haplotypes defined, it was then possible to systematically conduct genetic and genomic investigations of these *S*-haplotypes by themselves or in combination. Finally, a hypothesis for the genetic control of SI and SC was developed and verified in self-pollination and crossing experiments.

Literature Review

This literature review will focus on the genetic structure of sour cherry, the mechanism of gametophytic self-incompatibility (SI), and the proposed mechanisms for the breakdown of gametophytic SI.

The Origin of Sour Cherry

Sour cherry (*Prunus cerasus* L.) is an allotetraploid that was produced by the hybridization of the diploid sweet cherry (*P. avium*) and the tetraploid ground cherry (*P. fruticosa*) (Olden and Nybom, 1968). This hybridization likely occurred multiple times and in both directions, i.e. sweet cherry x ground cherry and ground cherry x sweet cherry, as evidenced by the presence of both *avium*-type and *fruticosa*-type cytoplasm in sour cherry (Brettin et al., 2000). The *fruticosa*-type cytoplasm is most prevalent but the *avium*-type cytoplasm has been detected in some selections, such as Cigány 59. The recurrent formation was aided by the overlapping distribution of sweet cherry and ground cherry in Eastern Europe.

The Genomic Structure of Cherry

The genome size of sweet cherry is small (2C = 0.7pg; 338 Mb), approximately double the size of the *Arabidopsis thaliana* genome (Arumuganathan and Earle, 1991). The relatively small amount of repetitive DNA sequences in the *Prunus* genome made it relatively easy for *Prunus* geneticists to identify a putative *pollen-S* gene (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003). Researchers working with more traditional model systems such as tomato, petunia and tobacco were overwhelmed by the highly repetitive nature of the Solanaceous *S*-locus (Coleman and Kao, 1992). However, the inability to transform *Prunus* species makes it impossible to conduct the necessary gain-of-function and loss-of-function transformation experiments to prove that a putative gene is the true *pollen-S* gene. The first genetic map of sour cherry, consisting of RFLP markers, was published in 1998 (Wang et al., 1998).

Although sour cherry is an allotetraploid that predominately exhibits disomic inheritance, it also exhibits a low frequency of tetrasomic inheritance (~5%) (Beaver et al. 1993) and quadrivalent pairing characteristic of an autotetraploid (Wang et al., 1998).

Prevalence of Self-Incompatibility

Self-incompatibility is one of the most prevalent mechanisms that prevents inbreeding and promotes out crossing. Although many forms of SI exist, the *S*-RNase-based gametophytic SI found in *Prunus* and the Solanaceae is one of the most widespread and economically important.

Phylogenetic analysis of the *S-RNase* gene suggests that the *S*-RNase-based gametophytic SI systems found in the Solanaceae, Scrophulariaceae and Rosaceae most likely share a common origin (Igic and Kohn, 2001). The most recent common ancestor of these three distantly related plant families is the ancestor to approximately 75 percent of all dicot families.

Gametophytic SI exists in many *Prunus* species, including several diploid species such as sweet cherry (Crane and Lawrence, 1929; Crane and Brown, 1937; Way, 1967), almond (*P. dulcis*: Socias i Company et al., 1976), Japanese apricot (*P. mu*me) and plum (*P. domestica*: Crane and Lawrence, 1929). A majority of the tetraploid sour cherry individuals are SC; however, SI types can be found in the center of diversity (Lech and Tylus, 1983; Redalen 1984). In addition, SI progeny can arise from crossing involving two SC sour cherry selections (Lansari and Iezzoni, 1990).

Gametophytic Self-Incompatibility in Sweet Cherry

Early classification of *S*-haplotypes and incompatibility groups was done solely via crossing experiments, which led to the identification of six *S*-haplotypes and 13 incompatibility groups (Mathews and Dow, 1969). Since the initial discovery that the stylar product of the *S*-locus in sweet cherry is an *S*-RNase (Bošković and Tobutt, 1996) the pace of *S*-haplotype classification and discovery has accelerated. Isoenzyme gels (Bošković and Tobutt, 2001), PCR profiles (Sonneveld et al., 2001; Wiersma et al., 2001; Choi et al., 2002; Sonneveld et al., 2003), RFLP analyses (Tao et al., 1999) and cDNA sequences (Tao et al., 1999; Wiersma et al., 2001) have been used to describe a total of 17 *S*-haplotypes.

The Mechanism of the RNase-Based Gametophytic Self-Incompatibility System

Gametophytic SI is controlled by a single multi-allelic locus, called the *S*-locus, which is hypothesized to contain at least two genes involved in determining the specificity of the SI interaction between pollen tubes and styles (de Nettancourt, 1977). Because of the presence of multiple genes within the *S*-locus, the term "haplotype" has been used to describe variants of the *S*-locus whereas the term "allele" describes variant of a given polymorphic gene at the *S*-locus (McCubbin and Kao, 2000). The growth of pollen tubes is arrested within the style if the pollen tube has a pollen-*S* product in common with either of the two *S*-RNases in the style.

The stylar component of gametophytic SI is a ribonuclease, called *S*-RNase, which was first discovered in the Solanaceae (Anderson, 1986; McClure et al., 1989), followed by the Rosaceae (Sassa et al., 1993) and Scrophulaliaceae (Xue et al., 1996).

These *S*-RNases are expressed in the transmitting tract of styles and cause the degradation of incompatible pollen tubes. *S*-RNases enter the pollen tubes of both compatible and incompatible pollen (Luu et al., 2000) but only degrade rRNA of incompatible pollen (McClure et al., 1990). Whether the *S*-RNase is activated in incompatible pollen tubes or inactivated in compatible pollen tubes is unknown, although the recent discovery that the *pollen-S* gene is an F-box gene (see below) suggests that the *S*-RNase in compatible pollen tubes is quickly degraded by the ubiquitin/proteasome proteolytic pathway (Ushijima et al., 2003). Gain-of-function and loss-of-function experiments have shown that *S*-RNase is necessary and sufficient for a style's ability to reject pollen (Lee et al., 1994; Murfett et al., 1994). In addition, mutation analyses have shown that RNase function is necessary for the rejection of self-pollen (Huang et al., 1994).

The *S-RNase* gene is composed of five conserved regions and two or one hypervariable regions in the Solanaceae (Ioerger et al., 1991) and Rosaceae (Ushijima et al., 1998), respectively. HVa and HVb, the two hypervariable regions in Solanaceous *S*-RNases, are exposed on the surface of the *S*-RNase protein (Ida et al., 2001) and under positive selection (Ishimizu et al., 1998), suggesting that they are likely the determinants of *S*-RNase specificity and may interact directly with the pollen-*S* product. Matton et al. (1997) provided functional evidence by changing the specificity of an *S*-RNase simply by swapping the HVa and HVb domains with those from a different *S-RNase* allele. Rosaceous *S-RNases* only have a single hypervariable region (RHV), which corresponds to the HVa from the Solanaceous *S-RNases* (Ushijima et al., 1998).

The *S-RNase* gene in all Solanaceous and Rosaceous species, including *Malus* species and *Pyrus* species but not *Prunus* species, contains a single intron (Igic and Kohn, 2001). The *S-RNase* gene from *Prunus* species contains a second intron near the 5' end of the gene. The lengths of these introns vary greatly between *S*-haplotypes, making them very useful for *S-RNase* genotyping (Tao et al., 1999; Wiersma et al., 2001).

The pollen component of gametophytic SI has, until recently, remained elusive. Recently, several candidate genes have been hypothesized to be the *pollen-S* gene. In each case, the candidate gene is a pollen-specific F-box gene that is located in the *S*-locus. The first report of an F-box gene, named *S*-locus F-box gene (*SLF*), was from *Antirrhinum* (Lai et al., 2002), which was later demonstrated to interact with the *S*-RNase in a haplotype-specific manner (Qiao et al., 2004). Similar *SLF* genes were isolated from *Prunus mume* (Entani et al., 2003). A different F-box gene, named *S*-haplotype-specific F-box gene (*SFB*), was isolated from *P. dulcis* (Ushijima et al., 2003) and showed higher levels of polymorphism. Transformation is not possible in these species, making it impossible to conduct the necessary gain-of-function and loss-of-function transformation experiments to prove that one of these F-box genes is the true *pollen-S* gene. However, correlation of mutations in the *SFB* with a loss-of-function of the pollen-*S* product has provided strong evidence that *SFB* is the *pollen-S* gene (see "Proposed Mechanisms for the Breakdown of gametophytic SI"; Ushijima et al., 2004). More recently, an F-box gene from *Petunia* (*PiSLF*) was shown via transformation to be sufficient for controlling the *S*-haplotype specific interaction with the style indicating that it is the *pollen-S* gene (Sijacic et al., 2004).

The finding that the pollen-determinant is an F-box protein suggests a possible mechanism for the interaction between the *S*-RNase and the pollen-*S* product. F-box proteins are components of SCF complexes, which regulate protein degradation in the ubiquitin / proteasome proteolytic pathway (Deshaies, 1999). The F-box protein acts as a protein receptor for the SCF, allowing the polyubiquitination and eventual degradation of the protein by the 26S proteasome. Thus, SFB might form a complex with SCF that polyubiquitinates all non-self *S*-RNases, thus causing their destruction. SFB might interact with its cognate *S*-RNase in a different manner to prevent polyubiquitination and allow it to remain active (Ushijima et al., 2003).

SFB is composed of many conserved residues, including a conserved region in the N-terminus that makes up the F-box motif, two variable regions, and two hypervariable regions, HVa and HVb (Ikeda et al., 2004). It is likely that the two hypervariable regions play a role in the *S*-haplotype-specific interaction with cognate *S*-RNases.

Proposed Mechanisms for the Breakdown of Gametophytic SI

SI is believed to be the ancestral state in as many as 75 percent of dicot families (Igic and Kohn, 2001). However, SI is not observed in all of these species, suggesting the repeated breakdown of SI to create SC individuals, populations or species. There are four main ways in which SI can breakdown. These four mechanisms are described below.

One way in which SC can arise in SI plants is through the mutation of the *S-RNase*, the pistil determinant of the haplotype-specific rejection of pollen. These mutations may either disrupt the function or the expression of the *S*-RNase in the styles.

Huang et al. (1994) were able to construct mutant *S-RNase* genes containing amino acid substitutions at the RNase active sites. These mutations prevented function of the *S*-RNase. Similar mutations in the wild would make the *S*-RNase non-functional, thus resulting in the inability to reject pollen containing the cognate pollen-*S* product. Yamane et al. (2003) reported the presence of a ~2600 bp insertion in the putative promoter region of the sour cherry S_{6m}-*RNase*. This insertion prevented expression of the S_{6m}-RNase in styles; however, it had no effect on the expression or function of the *pollen-S* gene. Both of these types of mutations specifically disrupt the ability of a style to recognize and degrade self-pollen. Pollen containing the S_{6m}-haplotype could still be rejected by styles containing a functional S_6-RNase.

Alternately, mutations could occur in the *pollen-S* gene, thus making it incapable of eliciting an SI reaction. Ushijima et al. (2004) reported structural mutations in *SFB*, the putative *pollen-S* gene, of two pollen-part mutants (PPMs) from *Prunus*. A four base pair deletion upstream from HVa and HVb in the S_4' pollen-part mutant in sweet cherry caused a frame-shift that results in defective SFB transcripts that lack the HVa and HVb. A 6.8 kb insertion upstream from the HVa and HVb of the S_f-*SFB* in *P. mume* caused a truncated SFB transcript which lacks the HVa and HVb regions. These mutations result in a loss of *pollen-S* gene function; however, the *S*-RNase remains functional and capable of rejecting pollen containing a corresponding functional *pollen-S* gene. Interestingly, despite the identification of several PPMs through mutant screens, no PPMs caused by either the loss of expression of the *pollen-S* gene or the expression of a mutant *pollen-S* gene have been observed in the Solanaceae (Pandey 1967; van Gastel and de Nettancourt, 1975; Golz et al., 1999; Golz et al., 2000). Instead, all identified PPMs were caused by

the duplication of the *pollen-S* gene (see "fourth class of mutations" for more details). This suggests that the loss of a functional pollen-*S* product does not cause a breakdown in SI in the Solanaceae.

The previous two mechanisms involve mutations of the *S*-locus linked specificity components of the SI reaction. However, mutations may occur in genes located outside of the *S*-locus and result in SC. McClure et al. (1999) detected a small asparagine-rich protein, named HT, which is critical for the SI reaction, although it is not involved in the initial interaction between the *S*-RNase and the pollen-*S* product. They report that diminished expression of the HT gene is correlated with the breakdown of SI. Mutations in "modifier genes", such as HT, can cause the disruption of the interaction between the *S*-RNase and pollen-*S* product of all *S*-haplotypes rather than just the loss-of-function of a single *S*-haplotype.

A fourth class of mutations that can cause the breakdown of SI is the duplication of the *pollen-S* gene. Two common mechanisms for the duplication of the *pollen-S* gene are the creation of centric fragments containing the *S*-locus (Brewbaker and Natarajan, 1960) and the increase in ploidy level. Crane and Lawrence (1931) were the first to associate an increase in ploidy level with a conversion from SI to SC while working with sweet cherry. Later, similar observations were made in *Pyrus communis* (Crane and Thomas, 1939), *Solanum* (Livermore and Johnstone, 1940), *Petunia* (Stout and Chandler, 1941), *Nicotiana alata* (Pandey 1968), and *Lycopersicon peruvianum* (de Nettancourt et al., 1974). It was also observed that the breakdown was unilateral in the Solanaceous plants, meaning that tetraploid styles maintained their ability to reject pollen from diploid plants but pollen from tetraploid plants could not be rejected by diploid or tetraploid

styles (Livermore and Johnstone, 1940; Stout and Chandler, 1941; Crane and Lewis, 1942; Pandey, 1968; de Nettancourt et al., 1974; Chawla et al., 1997). Stout and Chandler (1942) observed that this breakdown in polyploids occurred in heterozygous plants but not homozygous ones, suggesting that the breakdown only occurs in heteroallelic pollen. Lewis (1943) obtained similar results with *Oenothera* tetraploids and suggested that a competition between different pollen-*S* products within a single pollen tube causes the breakdown of SI in heteroallelic pollen but there is no competition between S-RNases in styles. More recent experiments involving *Petunia hybrida* support the hypothesis that heteroallelic pollen, but not pollen containing multiple copies of the same *pollen-S* gene, loses its SI phenotype (Entani et al., 1999). The breakdown of SI in heteroallelic pollen in the Solanaceae is so well accepted that transformation experiments were recently designed to take advantage of this phenomenon to confirm that *PiSLF* is the actual *pollen-S* gene (Sijacic et al., 2004).

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CHAPTER 2

REVISITING THE *S*-ALLELE NOMENCLATURE IN SWEET CHERRY (*PRUNUS AVIUM*) USING RFLP PROFILES

Abstract

Correct assignment of self-incompatibility alleles (*S*-alleles) in sweet cherry (*Prunus avium* L.) is important to assure fruit set in field plantings and breeding crosses. Until recently, only six *S*-alleles had been assigned. With the determination that the stylar product of the *S*-locus is a ribonuclease (RNase) and subsequent cloning of the *S-RNases*, it has been possible to use isoenzyme and DNA analysis to genotype *S*-alleles. As a result, numerous additional *S*-alleles have been identified; however, since different groups used different strategies for genotype analysis and different cultivars, the nomenclature contained inconsistencies and redundancies. In this study restriction fragment-length polymorphism (RFLP) profiles are presented using *Hin*dIII, *Eco*RI, *Dra*I, or *Xba*I restriction digests of the *S*-alleles present in 22 sweet cherry cultivars which were chosen based upon their unique *S*-allele designations and/or their importance to the United States sweet cherry breeding community. Twelve previously published alleles (S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_9, S_{10}, S_{11}, S_{12}, and S_{13}) could be differentiated by their RFLP profiles for each of the four restriction enzymes. Two new putative *S*-alleles, both found in 'NY1625', are reported, bringing the total to 14 differentiable alleles. We propose the adoption of a standard nomenclature in which the sweet cherry cultivars 'Hedelfingen' and 'Burlat' are S_3S_5 and S_3S_9, respectively. Fragment sizes for each *S*-allele/restriction enzyme combination are presented for reference in future *S*-allele discovery projects.

Introduction

Self-incompatibility (SI) is a common mechanism in flowering plants that prevents self-fertilization and promotes out-crossing (de Nettancourt, 1977). In gametophytic self-incompatibility (GSI), SI is determined by a single, multi-allelic locus, called the *S*-locus in which the compatibility of a cross is determined by the haploid genome of the pollen and the diploid genome of the pistil. In GSI, pollen tube growth is arrested if the pollen tube has a *S*-allele in common with one of the two *S*-alleles in the style. The *S*-locus is composed of multiple genes, one of which is an RNase (*S*-RNase) that is expressed only in the pistil. A second gene that is hypothesized to be expressed specifically in the pollen has yet to be determined from any GSI species.

Sweet cherry (*Prunus avium*) fertilization is controlled by a GSI system and therefore, knowledge of the *S*-allele composition of a tree is crucial for compatible pollination and fruit set. Knight (1969) named six *S*-alleles (S_1, S_2, S_3, S_4, S_5, and S_6) and categorized the cultivars into 13 compatibility groups and a Group O, which included cultivars that were SI but able to pollinate cultivars in all the other groups. As is the case with other reported GSI systems, the stylar *S*-allele component in SI members of the Rosaceae family is an *S*-RNase (Bošković and Tobutt, 1996; Broothaerts et al., 1995; Burgos et al., 1998; Ishimizu et al., 1996; Sassa et al., 1992; Sassa et al., 1996; Tao et al., 1997; Tao et al., 1999; Tomimoto et al., 1996; Ushijima et al., 1998; Yamane et al., 1999). In sweet cherry, RNase isoenzymes (Bošković et al., 1997) and cDNA sequences (Tao et al., 1999) have been associated with the stylar *S*-allele RNases.

Sour cherry (*P. cerasus* L.), which is a hybrid tetraploid species between sweet cherry and ground cherry (*P. fruticosa* Pall), consists of self-compatible and self-incompatible individuals; however, unlike sweet cherry, control of SI in sour cherry is unknown. Our long term goal is to determine the genetic control of SI in sour cherry (Yamane et al., 2001). We hypothesize that a similar RNase stylar component is present in sour cherry and that sweet and sour cherry may share common *S*-alleles. However, before embarking upon *S*-allele discovery in sour cherry, we needed to have a clear definition of the *S*-alleles that had been identified in sweet cherry.

A review of the sweet cherry *S*-allele literature revealed that potentially similar sweet cherry *S*-alleles had been assigned differing nomenclature (Bošković and Tobutt, 1996; Bošković et al., 1997; Choi et al., 2000; Knight, 1969; Schmidt and Timmann, 1997; Schmidt et al., 1999; Tao et al., 1999; Tehrani and Lay, 1991; Wiersma et al., 2001; Yamane et al., 2000). The confusion seems to originate from the initial incorrect classification of 'Hedelfingen' and 'Burlat' into Group VII with the assigned *S*-alleles, S_4S_5 (Knight, 1969). Since 'Hedelfingen' and 'Burlat' are cross compatible, they should have not been assigned to the same group. Tehrani and Lay (1991) recognized the *S*-allele misclassification of 'Hedelfingen' and assigned it to Group O. Bošković et al. (1997) proposed that 'Hedelfingen' contains the S_3- and S_5-alleles. Crosses done in Germany confirmed that 'Hedelfinger' ('Hedelfingen') was S_3S_5 while 'Burlat' was determined to contain neither S_4 nor S_5 (Schmidt and Timmann, 1997). 'Burlat' was assigned the *S*-alleles (S_3S_x) where S_x represented a novel *S*-allele (Schmidt et al., 1999). In this manuscript we propose the adoption of the S_3S_5 nomenclature for 'Hedelfingen' as proposed by Bošković et al. (1997), Schmidt and Timmann (1997) and Schmidt et al.

(1999). Therefore the objective of this research was to use restriction fragment-length polymorphisms (RFLPs) to characterize 12 sweet cherry *S*-alleles that had been published previously, as well as to characterize two unique *S*-alleles, and to propose the adoption of a standard nomenclature for these *S*-alleles. In addition, the *S*-genotypes of five new cultivars important to the United States sweet cherry breeding community are reported. Fragment sizes for each *S*-allele/restriction enzyme combination are also presented so this information can be used as a reference in future *S*-allele discovery projects.

Materials and Methods

Plant material

Young leaf tissue was collected from 22 sweet cherry cultivars in the spring. Leaves of 'Napoleon', 'Cavalier' and 'Gold' were collected at the Michigan State University Northwest Horticultural Research Station, Traverse City, Mich. Leaves of 'Charger', 'Gaucher', 'Inge', and 'Orleans 171' were kindly provided by K. Tobutt (East Malling, United Kingdom). Leaves of 'Early Rivers', 'Burlat', 'Schneiders', 'Seneca', 'Valera', 'Hedelfingen', 'Nadino', 'NY1625', and 'Guigne d'Annonay' were kindly provided by C. Choi and R. L Andersen (Geneva, N.Y.). Leaves of 'Noble' were kindly provided by B. Lay (Vineland, Ontario, Canada). Leaves of 'Chelan', 'Tieton', PMR-1, and PC-8007-2 were kindly provided by G. Lang (Prosser, Wash.). Leaves of 'Mona' (DPRU 2046) were obtained from the U.S. Department of Agriculture National Clonal Repository, Davis, Calif. Where possible, the leaf material was placed immediately on

dry ice. All frozen and fresh leaf material was lyophilized and stored at –20 °C until needed for DNA isolation.

DNA isolation

Total DNA was isolated from young leaves using the CTAB method described by Stockinger et al. (1996).

Genomic DNA blot analysis

Six micrograms of DNA was digested with either *Hin*dIII, *Eco*RI, *Dra*I, or *Xba*I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), run on 0.9 % agarose gel for 36 h at 30 V, and transferred to a nylon membrane (Hybond-N+, Amersham, Piscataway, N.J.) according to Wang et al. (1998). Polymerase chain reaction (PCR) amplified fragments of the S_6-RNase cDNA from sweet cherry (Tao et al., 1999) were used as the probe. Probes were radiolabelled with ^{32}P-dCTP (DuPont, Boston) using the random primer hexamer-priming method described by Feinberg and Vogelstein (1983). After hybridization at 60 °C for 16 h and high stringency washes (2 × 30 min with 2X SSC and 1% SDS followed by 2 × 30 min with 0.2X SSC and 0.5% SDS at 60 °C), bound radioactivity resulting from hybridizations was detected with X-ray film.

PCR amplification of *S*-alleles

PCR was performed on the sweet cherry cultivars using two primer pairs: SI-19 (5' CCA CCG ACC AAC TGC AGA GT 3') / SI-20 (5' TGG TAC GAT TGA AGC GT 3'), and SI-31 (5' STT STT GST TTT GCT TTC TTC 3') / SI-32 (5' CAT AGG CCA

TGR ATG GTG 3') which were designed by Wiersma et al. (2001). The PCR conditions were identical to those used by Wiersma et al. (2001). PCR reactions were run in a DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, Conn.), the resulting PCR mixtures were run on 0.9% agarose gels, and the DNA bands were visualized by ethidium bromide staining.

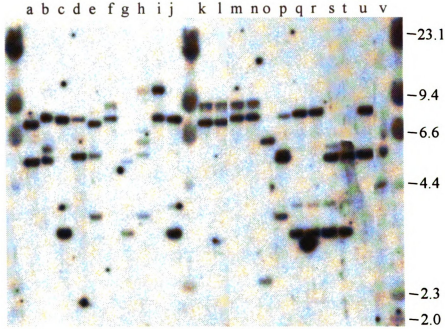
Results and Discussion

Twenty-two sweet cherry cultivars were analyzed by RFLP analyses using *Hin*dIII, *Eco*RI, *Dra*I, or *Xba*I restriction digestions (Figure 2.1). The four RFLP analyses gave consistent results, and it was possible to distinguish 14 different putative *S*-alleles with each of the four restriction enzymes (Table 2.1).

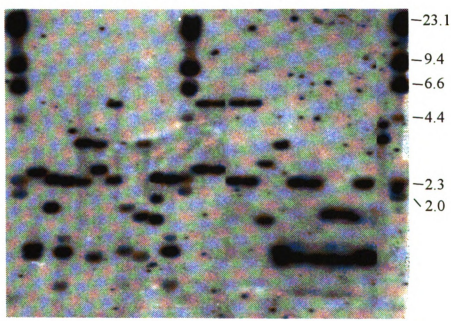
The *S*-genotypes of 'Early Rivers' (S_1S_2), 'Napoleon' (S_3S_4) and 'Gold' (S_3S_6) have not been questioned in the literature since first published (Knight, 1969) (Table 2.2). The RFLP fragment sizes for the S_1, S_2, S_3, S_4, and S_6 alleles following *Hin*dIII and *Eco*RI digests agree with those of Tao et al. (1999) with only slight variations due to enhanced resolution since in the present study the fragments were separated for a longer period of time on the agarose gel. In the *Hin*dIII digest, this enabled S_1 to be distinguished from S_3 while S_2, S_4, and S_6 were distinguished from one another, and in the *Eco*RI digest, this enabled S_1 to be distinguished from S_4. Each of these five *S*-alleles exhibits just one fragment with the exception of the S_4- and S_2- alleles, which exhibit two fragments following *Hin*dIII and *Dra*I digests, respectively (Table 2.1, Figure 2.1).

Figure 2.1. Genomic DNA blot analysis of 22 sweet cherry cultivars. Six micrograms of Genomic DNA was digested by (A) *Hin*dIII, (B) *Dra*I, (C) *Eco*RI, or (D) *Xba*I blotted to membrane and hybridized to the cDNA encoding S_6-RNase. Lambda/*Hin*dIII marker was used for size determination. (a) 'Early Rivers' (S_1S_2), (b) 'Napoleon' (S_3S_4), (c) 'Burlat' (S_3S_9), (d) 'Gold' (S_3S_6), (e) 'Charger' (S_1S_7) (f) 'Gaucher' (S_3S_5), (g) 'Inge' (S_4S_9), (h) 'Orleans 171' ($S_{10}S_{11}$), (i) 'Schneider' (S_3S_{12}), (j) 'Mona' (S_3S_9), (k) 'Seneca' (S_1S_5), (l) 'Valera' (S_1S_5), (m) 'Hedelfingen' (S_3S_5), (n) 'Nadino (S_3S_5) (o) 'NY1625' (S_uS_v), (p) 'Guigne d'Annonay' (S_2S_7), (q) 'Chelan' (S_3S_9), (r) 'Tieton' (S_3S_9) (s) 'PMR-1' (S_4S_9), (t) '8007-2' (S_4S_9), (u) 'Cavalier' (S_2S_3), and (v) 'Noble' (S_6S_{13}).

A *Hin*dIII

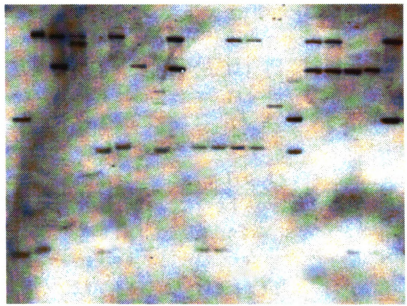


B *Dra*I



C *Eco*RI

a b c d e f g h j k l m n o p q r s t u



D *Xba*I

a b c d e f g h i j m p

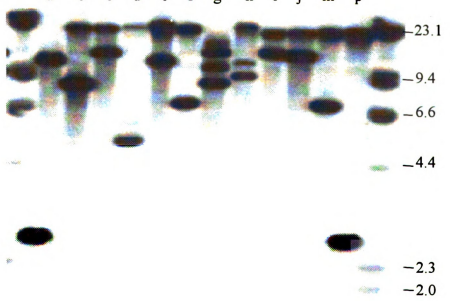


Table 2.1 Sizes of DNA restriction fragments for sweet cherry *S*-alleles used in this study

	Size (kb)			
S-allele	*Hin*dIII	*Eco*RI	*Dra*I	*Xba*I
1	8.7	1.5	2.5	13.0
2	5.6	4.4	0.8, 1.0	2.6
3	8.8	13.1	2.3	20.0
3 'Gaucher' [z]	8.8	13.1	2.3	20.0
4	5.6, 6.1	1.8	1.8	8.8
5	9.4	3.5	5.3	6.8
6	5.8	11.0	3.5	5.5
7	3.5, 5.8, 8.7	3.3, 6.0	0.8, 3.45	21.0
9	3.1	7.9	0.9	0.0, 16.0, 18.0
9 'Burlat' [y]	3.1, 4.0	7.9	0.6[v], 0.9, 1.2	15.0
10 or 11[x]	3.5, 5.8, 6.4, 6.6, 12.1	3.3, 5.0, 5.5	0.8, 1.6, 3.5	9.4, 13.0, 21.0
12	12.1	-	1.5, 1.9	16.0
13	4.6, 6.5	-	4.4	-
u or v[w]	2.5, 6.4	4.8	1.6, 2.7	-

[z] This *S*-allele in 'Gaucher' was originally thought to be a unique *S*-allele (S_8) (Bošković et al. 1997).

[y] This *S*-allele in 'Burlat' was originally thought to be a unique *S*-allele (S_x) (Schmidt et al., 1999).

[x] These are the two *S*-alleles in 'Orleans 171'. Restriction fragments for S_{10} and S_{11} were grouped together because it could not be determined which fragments corresponded to each *S*-allele.

[w] These are the two putative unique *S*-alleles in 'NY1625'. Restriction fragments for S_u and S_v were grouped together because it could not be determined which fragments corresponded to each *S*-allele.

Table 2.2 *S*-allele genotypes of 17 sweet cherry cultivars used in this study.

Cultivar	*S*-allele genotype	Other published nomenclature
'Early Rivers'	1,2 [z]	
'Napoleon'	3,4 [z]	
'Hedelfingen'	3,5 [y, x]	4,5 [z] ; 3,x [w] ; 3,15 [v]
'Nadino'	3,5 [x]	3,x [w]
'Seneca'	1,5	1,x [w]
'Valera'	1,5 [x, u]	1,x [w] ; 1,15 [v]
'Gold'	3,6 [z]	
'Charger'	1,7 [t]	
'Guigne d'Annonay'	2,7 [s]	2,z [w]
'Gaucher'	3,5 [s]	5,8 [t]
'Inge'	4,9 [t]	
'Orleans 171'	10,11 [t]	
'Schneiders'	3,12 [s]	3,13 [v] ; 3,y [w]
'Burlat'	3,9 [s]	3,x [x] ; 4,5 [z] ; 3,5 [w,v,r]
'Mona'	3,9	2,14 [v]
'Noble'	6,13 [s]	6,? [v] , 1,6 [w] .
'NY1625'	u,v	4,x [w]

[z] Knight (1969).

[y] Bošković and Tobutt (1996).

[x] Schmidt et al. (1999).

[w] Choi et al. (2000).

[v] Wiersma et al. (2001).

[u] Way (1968)

[t] Bošković et al. (1997).

[s] Bošković and Tobutt (2001).

[r] Tao et al. (1999).

As reported by Bošković et al. (1997), 'Charger' (S_1S_7) and 'Inge' (S_4S_9) each exhibit one new *S*-allele. These alleles, called S_7 and S_9, displayed from one to three unique fragments per allele following Southern hybridization (Table 2.1, Figure 2.1). PCR of the S_7 allele using the primer pair SI19/SI20 did not amplify any fragment; whereas, a fragment of 425 base pairs (bp) (similar to the S_2, S_9, and S_{12} alleles) was amplified when using the primers SI31/SI32 (Figure 2.2). S_9 produced one 745 bp amplification product and two fragments of 425 bp (similar to S_2, S_7, and S_{12} alleles) and 615 bp, respectively, when amplified with SI19/SI20 and SI31/SI32.

The presence of two unique *S*-alleles in 'Orleans 171' ($S_{10}S_{11}$) also agrees with that of Bošković et al. (1997). RFLP analysis of 'Orleans 171' produced either three or five fragments, depending on what restriction enzyme was used (Table 2.1, Figure 2.1) and the fragment patterns did not match that of any known *S*-alleles. It could not be determined which RFLP fragments represent the S_{10} versus the S_{11} alleles since differential cultivars, such as S_3S_{10} and S_3S_{11} are not available. Neither S_{10} nor S_{11} could be amplified using either of the PCR primer pairs, SI19/SI20 or SI31/SI32 (Figure 2.2). These results support the conclusion that 'Orleans 171' contains two unique *S*-alleles.

'Noble' was initially assigned the *S*-alleles S_1 and S_6 (Choi et al., 2000), but was later found to contain S_6 and a unique *S*-allele, which was temporarily named $S_?$ (Wiersma et al., 2001). Restriction digestion of 'Noble' with either *Hin*dIII or *Dra*I produced fragments that did not match any found in other cultivars (Table 2.1, Figure 2.1). Bošković and Tobutt (2001) named this allele S_{13}. In order to remain consistent with the European nomenclature, we suggest retaining the genotype of S_6S_{13} for 'Noble'.

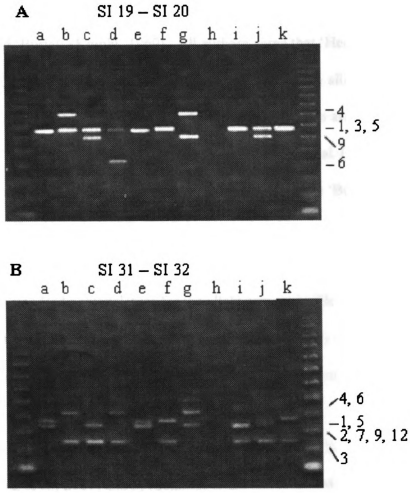


Figure 2.2. PCR analysis of *S*-alleles from sweet cherry. Genomic DNA was PCR amplified using two primer sets: (A) SI19/20 and (B) SI31/32. A 123 bp DNA ladder was used for size determination. (a) 'Early Rivers' (S_1S_2), (b) 'Napoleon' (S_3S_4), (c) 'Burlat' (S_2S_9), (d) 'Gold' (S_2S_6), (e) 'Charger' (S_1S_7) (f) 'Gaucher' (S_2S_5), (g) 'Inge' (S_4S_9), (h) 'Orleans 171' ($S_{10}S_{11}$), (i) 'Schneider' (S_3S_{12}), (j) 'Mona' (S_3S_9), and (k) 'Hedelfingen' (S_3S_5).

The *S*-genotypes of the other cultivars in Table 2.2 have been more difficult to determine and much of this difficulty can be traced to the initial misclassification of 'Hedelfingen' and 'Burlat' (Knight, 1969). All authors agree that 'Hedelfingen' and 'Nadino' have a S_3 allele and that 'Seneca' and 'Valera' have an S_1 allele. It is the second common allele present in these four selections that has been assigned conflicting nomenclature. Choi et al. (2000) called this allele S_x. Wiersma et al. (2001) have named this 'Hedelfingen' allele S_{15}. Both groups of researchers called the 'Burlat' allele S_5. However, both Bošković et al. (1997) and Schmidt et al. (1999) called the allele in 'Hedelfingen' S_5 prior to either of these publications. Therefore, we recommend that S_5 be adopted as the standard nomenclature for the allele present in 'Hedelfingen' and also in 'Nadino', 'Seneca', 'Valera', and 'Gaucher' (Table 2.2). This S_5 allele exhibited just one fragment when digested with any of the four restriction enzymes (Table 2.1, Figure 2.1).

The unique *S*-allele present in 'Burlat' that was called S_5 (Choi et al., 2000; Tao et al., 1999; Wiersma et al., 2001) and S_x (Schmidt et al., 1999) should be renamed. Recently, this *S*-allele has been sequenced, and found to have an identical sequence to the S_9 allele found in 'Inge' (T. Sonneveld, personal communication). Therefore, we propose the *S*-allele nomenclature for 'Burlat' be S_3S_9. The RFLP profiles of the S_9 allele in 'Burlat' are similar to the profiles of the S_9 allele from 'Inge' following digestion with *Hin*dIII, *Eco*RI, and *Dra*I with the only differences being the presence of extra faint bands in the 'Burlat' allele when digested with *Hin*dIII or *Dra*I (Table 2.1, Figure 2.1). This can be explained by differential length of exposure or differing amounts of DNA in the digestion reaction. However, the profiles of the S_9 alleles in 'Burlat' and 'Inge' are

significantly different after digestion with *Xba*I. This presents a shortcoming of RFLP analysis for *S*-allele genotyping. If the restriction enzyme cut site(s) resulting in the polymorphism(s) are not within the *S-RNase*, but instead are located in the regions flanking the *S-RNase*, it is possible that identical *S*-alleles could exhibit different RFLP fragments.

'Mona' used in this research has the same genotype as 'Burlat' (S_3S_9) (Figures 2.1 and 2.2). This contradicts the finding by Wiersma et al. (2001) that the *S*-genotype of 'Mona' is S_2S_{14}. The probable explanation is that the 'Mona' trees from which the leaves were collected for each study (the USDA Clonal Repository, Davis, Calif. and Vineland, Ontario, Canada, respectively) were not the same cultivar. These conflicting results reinforce the importance of not only knowing the purported cultivar, but also the source in case identities are mistaken across locations.

Using PCR data, Choi et al. (2000) determined that 'Guigne d'Annonay' contained an allele that differed from any previously reported *S*-allele and thus named it S_z. The *Hin*dIII, *Eco*RI, *Dra*I, and *Xba*I RFLP analyses all suggest that the S_z allele in 'Guigne d'Annonay' is the same as the S_7 allele in 'Charger' (compare lanes e and p on Figure 2.1). Therefore, we propose that the actual *S*-genotype of 'Guigne d'Annonay' should be S_2S_7 (Table 2.2). Similarly, Yamane et al. (2000) used RFLP analyses and *S*-RNase patterns on 2D-PAGE to discover a novel *S*-allele in the cultivar, Hinode (syn. Early Purple). Based on their RFLP patterns after digestion with *Eco*RI or *Hin*dIII restriction enzymes, this novel *S*-allele also appears to be S_7.

'Schneiders' has been reported to contain an additional unique *S*-allele, named S_y by Choi et al. (2000), S_{13} by Wiersma et al. (2001), and S_{12} by Bošković and Tobutt

(2001). In order to remain consistent with the European nomenclature, we suggest retaining the genotype of S_3S_{12} for 'Schneiders'. S_{12} exhibited two fragments following *Dra*I digest and one fragment following *Hin*dIII and *Xba*I digests (Table 2.1).

Bošković et al. (1997) presented the RNase isoenzyme patterns for five new *S*-alleles: S_7, S_8, S_9, S_{10}, *and* S_{11}. Whereas our RFLP and PCR analyses support the conclusion that S_7, S_9, S_{10}, and S_{11} are unique *S*-alleles, these analyses did not provide evidence that S_8, which is supposedly present in 'Gaucher', is truly a new *S*-allele. None of the four restriction digests were able to detect a difference between S_3 and S_8 (Table 2.1, Figure 2.1). PCR amplification using the primer pairs SI19/SI20 and SI31/SI32 was also unable to differentiate between S_3 and S_8 (Figure 2.2). Both the S_3 and S_8 alleles produced a fragment of 825 or 300 bp when amplified with SI19/SI20 or SI31/SI32, respectively. Recent cloning and sequencing of the S_8 allele has shown that the sequence for the S_3 and S_8 *RNases* are identical (Sonneveld et al., 2001). Therefore, the *S*-allele designation for 'Gaucher' should be S_3S_5 rather than S_5S_8.

The RFLP patterns from the *Hin*dIII, *Eco*RI, and *Dra*I RFLP analyses suggest that the selection 'NY1625' contains two *S*-alleles represented by one fragment each that are not found in any other cultivar (lane "o" in Figure 2.1A, B and C; Table 2.1). PCR amplification of 'NY1625' using the primer pair SI19/SI20 confirms the presence of at least one unique *S*-allele in 'NY1625' as a single 670 bp fragment (data not presented) was produced, which is different from all known *S*-alleles. However, using PCR, Choi et al. (2000) reported that the *S*-genotype for this selection was S_4S_x (S_4S_5, using the proposed nomenclature), which would be expected given the parentage of 'NY1625' ('Hedelfingen' S_3S_5 x 'Emperor Frances' S_3S_4) (Choi, 1999). It is possible that the DNA

sample contained some polysaccharide that altered the efficiency of the restriction digests and PCR amplifications. However, it is also likely that the DNA used in these RFLP analyses was mistakenly not collected from 'NY1625'. We propose that these two *S*-alleles be temporarily named S_u and S_v until crossing data confirms that they are indeed unique *S*-alleles.

Table 2.3 shows the *S*-genotypes of four new selections from Washington State University and a new cultivar from Michigan. Both 'Chelan' and 'Tieton' are S_3S_9, while PMR-1 and PC-8007-2 are S_4S_9. Choi et al. (2000) reported that the *S*-genotype of 'Chelan' is identical to that of 'Burlat', which agrees with the RFLP data. The other three cultivars/selections have not been genotyped previously. PMR-1 has been confirmed to be SI (G. Lang, personal communication), thus, this breeding line must contain S_4 as opposed to the fertile *S*-allele, $S_{4'}$. The pedigree of PC-8007-2 suggests that it contains the $S_{4'}$ allele; however, crossing data suggests that it is SI. These conflicting results make it currently impossible to determine if PC-8007-2 contains S_4 or $S_{4'}$. Currently, there are no molecular methods to differentiate between S_4 and $S_{4'}$. Therefore, more crossing data are needed to determine if PC-8007-2 is SI or SC. The genotype of 'Cavalier' is S_2S_3. The pedigrees for each of these new cultivars confirm the *S*-genotypes determined by RFLP and listed in Table 2.3.

PCR has been used by several researchers to differentiate between *S*-alleles. Tao et al. (1999) was able to differentiate between six *S*-alleles using two primer sets. Likewise, Wiersma et al. (2001) could distinguish nine *S*-alleles using two primer sets, with the aid of restriction digestions of the PCR-amplified products. However, these

Table 2.3. *S*-genotypes for five Washington State University and Michigan sweet cherry selections.

Selection	*S*-allele genotype
'Chelan'	3,9
'Tieton'	3,9
PMR-1	4,9
PC-8007-2	4,9
'Cavalier'	2,3

studies did not examine all known *S*-alleles. In the current study, additional *S*-alleles were examined. Some of the new *S*-alleles produced amplification fragments that were the same size as those produced by previously studied *S*-alleles. For example, the SI31/SI32 primer pair could not distinguish between S_2, S_7, S_9, and S_{12} (Figure 2.2). In addition, neither primer set could amplify S_{10} or S_{11}. It is possible that restriction digestion of the PCR products would allow for differentiation between S_2, S_7, S_9, and S_{12}; however, the digestions would be of no use to identify S_{10} and S_{11}, since they are both null alleles. As more *S*-alleles are discovered, the number of null alleles and confounding alleles is likely to increase. Nonetheless, PCR is still a useful tool for obtaining quick confirmation of what *S*-alleles are in the progeny of a cross between known parents. In addition, once new *S*-alleles are discovered and cloned, allele-specific primers could be produced which would allow differentiation of all *S*-alleles by PCR for genotyping projects. However, for *S*-allele discovery projects, a more powerful method for differentiating between *S*-alleles is needed. The potential of RFLP for discovery and identification of new *S*-alleles has been demonstrated by the fact that all *S*-alleles can be distinguished based on their unique banding patterns after digestion with any of the four restriction enzymes used in the present study. However, when interpreting RFLP data, it must be taken into consideration that the *S*-allele probe is hybridizing to fragments that include regions flanking the *S-RNase*. If the flanking regions of two identical *S-RNases* differ for their restriction enzyme cut sites, it is possible that different RFLP profiles may be observed in the cultivars even though the *S*-alleles are not unique, leading to the incorrect assumption that a new *S*-allele has been discovered.

Correct identification of *S*-allele genotype is critical for determining pollen compatibilities for field plantings and breeding crosses. Less than 5 years ago, the presence of only six *S*-alleles had been reported in sweet cherry (Bošković and Tobutt, 1996; Schmidt and Timmann, 1997). Since the stylar component of the *S*-locus in sweet cherry is believed to be an *S*-RNase, new methods are available to discover new *S*-alleles and *S*-allele discovery has proceeded at a rapid pace. It would not be surprising if many more unique *S*-alleles exist in natural populations of sweet cherry, since other plant species having a GSI system have been reported to have a very large number of *S*-alleles. For example, 37 and 39 different *S*-alleles were reported for evening primrose (*Oenothera organensis* Munz) (Emerson, 1939) and white clover (*Trifolium repens* L.) (Atwood, 1944), respectively.

The first six *S*-alleles from sweet cherry were discovered using crossing data. Since then, all of the subsequent *S*-alleles have been identified using molecular techniques. However, proper verification that each *S*-allele is unique requires crossing data. Unfortunately, as the number of unique *S*-alleles in sweet cherry increases, it becomes more cumbersome to perform all of the necessary diallele crosses. As a result, it is important to have available a molecular technique that can differentiate reliably between all unique *S*-alleles. Perhaps the most accurate method to verify the uniqueness of *S*-alleles, besides crossing, is to compare the amino acid sequences of each *S*-allele. However, sequences have not been reported for all known *S-RNases*. The complete amino acid sequences of S_1 (AB028153), S_2 (AJ298311), S_3 (AB010306), S_4 (AB028154), and S_6 (AB010305) can be found on GenBank (http://www.ncbi.nlm.nih.gov/). Until all amino acid sequences are reported, we propose

the use of RFLP analysis to investigate the uniqueness of an *S*-allele. For this to be effective, it is necessary that the RFLP patterns for new *S*-alleles be published for comparison. For example, Table 2.1 presents the fragment sizes for each of the *S*-alleles as determined by RFLP analysis with *Hin*dIII, *Eco*RI, *Dra*I, or *Xba*I restriction enzymes. When a putative new *S*-allele is discovered, the researcher can compare its fragment sizes with those presented in Table 2.1 to determine if the *S*-allele is likely a new allele, or if it matches an already existing *S*-allele. The alternative to comparing data with that presented in this table is to include all known *S*-alleles as controls, but as the number of unique *S*-alleles increases, this will get more cumbersome. This strategy of comparing RFLP fragments with fragments produced for known *S*-alleles in sweet cherry was used to propose five new *S*-alleles in sour cherry (Yamane et al., 2001).

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CHAPTER 3

SELF-COMPATIBILITY AND INCOMPATIBILITY IN TETRAPLOID SOUR CHERRY (*PRUNUS CERASUS* L.)

Abstract

Gametophytic self-incompatibility (GSI) typically "breaks down" due to polyploidy in many Solanaceous species, resulting in self-compatible (SC) tetraploid individuals. However, sour cherry (*Prunus cerasus* L.), a tetraploid species resulting from hybridization of the diploid sweet cherry (*P. avium* L.) and the tetraploid ground cherry (*P. fruticosa* Pall.), is an exception, consisting of both self-incompatible (SI) and SC individuals. Since sweet cherry exhibits GSI with 13 *S*-ribonucleases (*S*-RNases) identified as the stylar *S*-locus product, the objectives were to compare sweet and sour cherry *S*-allele function, *S-RNase* sequences and linkage map location as initial steps towards understanding the genetic basis of SI and SC in sour cherry. *S-RNases* from two sour cherry cultivars that were the parents of a linkage mapping population were cloned and sequenced. The sequences of two *S-RNases* were identical to those of sweet cherry *S-RNases*, whereas three other *S-RNases* had unique sequences. One of the *S-RNases* mapped to the *Prunus* linkage group 6, similar to its location in sweet cherry and almond, whereas two other *S-RNases* were linked to each other but were unlinked to any other markers. Interspecific crosses between sweet and sour cherry demonstrated that gametophytic SI exists in sour cherry and that the recognition of common *S*-alleles has been maintained in spite of polyploidization. It is hypothesized that self-compatibility in sour cherry is caused by the existence of non-functional *S-RNases* and *pollen S*-genes that may have arisen from natural mutations.

Introduction

Self-incompatibility (SI) is a common evolutionary strategy used by flowering plants to prevent self-fertilization and promote out-crossing (de Nettancourt 1977). In gametophytic self-incompatibility (GSI), SI is determined by a highly multi-allelic locus, called the *S*-locus, in which the compatibility of a cross is determined by the haploid genome of the pollen and the diploid genome of the pistil. In GSI, pollen tube growth is arrested if the pollen tube has an *S*-allele in common with one of the two *S*-alleles in the style. The *S*-locus has been classically described as a complex containing multi-allelic genes expressed by the pollen and style and tight linkage between these components. Because of the presence of at least two multi-allelic genes, the term "haplotype" has been used to describe variants of the *S*-locus and the term "allele" to describe variants of a given polymorphic gene at the *S*-locus (McCubbin and Kao 2000). In the Solanaceae and the Rosaceae, the gene controlling the pistil's self-incompatibility response is a ribonuclease (*S*-RNase) which is expressed only in the pistil (McClure et al. 1989; Sassa et al. 1992; Lee et al. 1994; Murfett et al. 1994; Broothaerts et al. 1995; Boskovic and Tobutt 1996; Ishimizu et al. 1996; Sassa et al. 1996; Tomimoto et al. 1996; Tao et al. 1997; Burgos et al. 1998; Ushijima et al. 1998; Tao et al. 1999; Yamane et al. 1999). A second gene that is hypothesized to be expressed specifically in the pollen has yet to be determined from any GSI species. Additional modifier genes have also been demonstrated to be required for normal SI function (McClure 1999).

GSI present in diploid species has been observed to 'break down' due to polyploidy with the tetraploid relatives frequently self-compatible (SC) (Livermore and

Johnstone 1940; Stout and Chandler 1942; Crane and Lewis 1942; Brewbaker 1954; Pandy 1968; de Nettancourt et al. 1974; Ueda and Akimoto 2001). To explain this phenomenon, Lewis (1947) proposed that pollen containing two different *S*-loci loses its SI phenotype resulting in SC polyploid individuals. Evidence obtained from recent research in Solanaceous species supports this theory (Chawla et al. 1997; Entani et al. 1999; Golz et al. 1999; Luu et al. 2001). In contrast, the GSI diploid sweet cherry (*Prunus avium* L., 2n=2x=16) and the tetraploid sour cherry (*P. cerasus* L., 2n=4x=32) represent a natural diploid – tetraploid series where the tetraploid individuals can be either SI or SC.

Sweet cherry and the tetraploid ground cherry (*P. fruticosa* Pall., 2n=4x=32) are believed to be the parental species that gave rise to sour cherry multiple times via unreduced gametes from sweet cherry (Olden and Nybom 1968; Iezzoni and Hancock 1984; Brettin et al. 2000). Although the vast majority of sour cherry cultivars are SC, numerous SI cultivars exist in Eastern Europe, the center of diversity (Lech and Tylus 1983; Redalen 1984a, 1984b; Lansari and Iezzoni 1990; Iezzoni et al. 1990). However, the SI phenotype is not limited to landrace cultivars as SI sour cherry selections can result from crosses between two SC sour cherry parents (Lansari and Iezzoni 1990). For example, a sour cherry linkage mapping population generated by crossing two SC sour cherry cultivars, 'Rheinische Schattenmorelle' (RS) x 'Érdi Bőtermő' (EB), segregates for SI and SC (Wang et al. 1998). Since any successful new sour cherry cultivar would have to be SC to avoid the production problems associated with providing pollinator trees, our goal was to determine the genetic basis of SI and SC in sour cherry to increase the likelihood of obtaining SC progeny in our sour cherry breeding program.

Sweet cherry exhibits classical gametophytic self-incompatibility with 13 *S*-RNases identified and validated in crossing experiments (Matthews and Dow 1969; Boskovic and Tobutt, 1996; Choi et al. 2000; Boskovic and Tobutt 2001; Hauck et al., 2001; Wiersma et al. 2001). In contrast, there is only one study of SI in sour cherry that takes advantage of the ability to determine putative *S-RNase* genotypes. Yamane et al. (2001) recently cloned two *S-RNases* from 'EB'; one matched the S_4*-RNase* previously cloned from sweet cherry and the second *S-RNase* was a novel *S-RNase* not previously identified in sweet cherry. RFLP and PCR analysis of *S-RNase* alleles in a set of sour cherry cultivars identified an additional four *S-RNases* that are presumably identical to previously identified sweet cherry *S-RNases* and an additional three putative novel *S-RNases*. The 'RS' and 'EB' mapping parents had the putative *S-RNase* designations, $S_aS_bS_cS_6$ and $S_aS_4S_{6m}$, respectively. Yamane et al. (2001) further compared the *S-RNase* allele composition of six SI with seven SC selections and found that all SI selections, similar to the SC selections, contained three or four different putative *S*-RNase alleles. This suggests that heteroallelic pollen alone may be insufficient to cause SC in tetraploid sour cherry.

Due to the evolutionary relatedness of sweet and sour cherry, and the potentially on-going gene flow between the two species, it is not surprising that *S-RNases* presumably identical to those found in sweet cherry were identified in sour cherry (Yamane et al. 2001). We further investigated SI and SC in sour cherry by taking advantage of the potential commonalities between these two species. The inheritance and linkage map locations of the putative *S-RNases* from 'RS' and 'EB' could also be determined and compared with information from other *Prunus* species. In sweet cherry

and almond (*Prunus dulcis*), the *S*-locus has been mapped to the end of the *Prunus* linkage Group 6 (Ballester et al. 1998; K. Tobutt, pers. comm.).

Our objectives were: (1) to determine if the *S*-alleles that appeared to be common between sweet and sour cherry exhibited the expected recognition reactions in the styles by making inter-specific crosses, (2) to determine the amino acid sequences of the *S*-RNases in 'EB' and 'RS' and compare them with previously sequenced sweet cherry *S*-RNases, and (3) to determine the sour cherry linkage map locations for the *S-RNase* loci.

Materials and Methods

Plant material

The two SI sour cherry cultivars 'Crisana' and 'Tschernokorka' were chosen for pollination with sweet cherry cultivars based on previous examination of their *S-RNase* profiles using PCR and RFLP analyses (Yamane et al. 2001). 'Crisana' contains three different *S-RNases*, one of which is presumably present in double dose. Two of the *S-RNases* produce RFLP and PCR fragment profiles identical to the S_1- and S_4-alleles in sweet cherry. The third *S-RNase* is not similar to any sweet cherry *S-RNase*, and is called S_d. 'Tschernokorka' also contains three different *S-RNases*, only one of which is identical to a sweet cherry *S-RNase* (S_9). The other two *S-RNases* are named S_a and S_c. The three sweet cherry cultivars used were 'Schmidt' (S_2S_4), 'Rainier' (S_1S_4) and 'Sato Nishiki' (S_3S_6). 'Crisana', 'Tschernokorka', and 'Schmidt' are maintained at MSU's Clarksville Horticultural Experimental Station (CHES), Clarksville, Mich. 'Rainier' is

maintained at the North West Horticultural Research Station, Traverse City, Mich, while 'Sato Nishiki' pollen was collected from trees growing at the Experimental Farm of Kyoto University, Kyoto, Japan, dried and frozen at –20 C. All pollen samples were tested to verify pollen viability prior to the crossing experiments as described by Brewbaker and Kwack (1963).

A pseudo-testcross mapping population consisting of 85 progeny from the cross between two sour cherry cultivars, 'Rheinische Schattenmorelle' ('RS') x 'Érdi Bőtermő' ('EB') (Wang et al. 1998) was maintained at CHES.

Analysis of pollen tube growth

Eight inter-specific crosses were performed and pollen tube growth was observed to determine whether the crosses were compatible or incompatible. Styles from each of the sour cherry cultivars ('Crisana' and 'Tschernokorka') were pollinated with pollen from the sweet cherry cultivars, 'Sato Nishiki' or 'Rainier'. Styles from the sweet cherry cultivars ('Rainier' and 'Schmidt') were pollinated with 'Crisana' and 'Tschernokorka' pollen. Pollination tests were performed as described by Lansari and Iezzoni (1990) with slight modifications. Pollen from newly opened flowers was collected from each of the pollen parents. Pollen was dried for 24 h. For each of the stylar parents, a branch with flowers at the balloon stage was brought into the laboratory and twenty flowers were emasculated. All other flowers were removed. Ten emasculated flowers were hand pollinated when receptive (24 h after emasculation) with each of the pollen sources. The pollinated pistils were collected 72 h after pollination, placed in fixing solution [(1 chloroform : 3 (95%) ethanol : 1 glacial acetic acid) (v/v)] for 24 h, transferred into 100%

ethanol, and stored at 4 °C until used for observation. The pistils were washed thoroughly under running tap water, incubated in 10 N NaOH for 5 to 6 h to soften the tissues, and soaked in 0.1 % aniline blue solution with 33 mM K_3PO_4 for 1 h to fluorescently stain the pollen tubes. Pollen tubes were observed by ultraviolet fluorescent microscopy (BX60, Olympus, Tokyo, Japan).

To determine whether the 77 flowering progeny of the 'RS' x 'EB' mapping population progeny were SI or SC, *in vitro* pollination tests were performed as described above. Styles from each of the progeny were pollinated with either self-pollen or with pollen from a collection of several unrelated sour cherry cultivars (out-cross pollen).

cDNA Cloning of *S-RNases*

Total RNA was isolated from 'RS' styles as described by Tao et al. (1999). One microgram of total RNA was used for first strand cDNA synthesis by SUPER SCRIPT II RT (Life Technologies, MD) with an Adp-dT primer (5'-CGACGTTGTAAAACGACGGCCAGTTTTTTTTTTTTTTT -3') that consisted of M13-20 sequence primer and oligo $(dT)_{16}$ (Tao et al. 1999). Pru-T2 primer (5'-TSTTSTTGSTTTTGCTTTCTTC -3') (Tao et al. 1999) derived from the cDNA sequence corresponding to the signal peptide sequence of *S*-RNases of sweet cherry was used in 3' rapid amplification of cDNA ends (3' RACE) with M13-20 primer as the adapter primer. PCR was performed using a program of 30 cycles at 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 7 min. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 400 nM each of primers,

template cDNA equivalent to the amount synthesized from 0.1 μg of the total RNA, and 1 U of Ex Taq polymerase (TaKaRa Shuzo Co, Shiga, Japan) in a 50 μL reaction volume. The PCR products were subcloned into the T-A cloning vector (pGEM-T Easy Vector System; Promega, Madison, Wisc.). DNA was sequenced using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The deduced amino acid sequences of four different cDNAs from 'RS' obtained in this study and two different cDNAs from 'EB' (Yamane et al. 2001) were aligned using Clustal X (Thompson et al. 1997).

DNA isolation and Southern analyses from the parents and progeny in the linkage mapping population

Young, unfolded leaves were collected from the parents and the progeny, placed on dry ice, stored at -80 C overnight and then lyophilized for 48 h. DNA was isolated using the CTAB method described by Stockinger et al. (1996). 'EB', 'RS', and the linkage mapping progeny were evaluated using Southern blotting following *Hin*dIII digest which had previously been demonstrated to differentiate all the 'EB' and 'RS' *S-RNases* (Yamane et al. 2001).

Six μg of DNA for both parents and 85 progeny was digested with *Hin*dIII (Boehringer Mannheim Biochemicals, Indianapolis), run on a 0.9 % agarose gel for 36 h at 30 V, and transferred to a nylon membrane (Hybond-N+, Amersham) according to Wang et al. (1998). PCR amplified fragments of the S_6-*RNase* cDNA from sweet cherry (Tao et al., 1999) were used as the probe. Probes were radiolabelled with ^{32}P-dCTP

(DuPont, Boston) using the random primer hexamer-priming method described by Feinberg and Vogelstein (1983). After hybridization at 60°C for 16 hours and high stringency washes (2 × 30 min with 2 X SSC and 1 % SDS followed by 2 × 30 min with 0.2 X SSC and 0.5 % SDS at 60 °C), radioactive signal was detected on X-ray films.

Inheritance and linkage analysis

Segregation of the *S*-alleles that were present in one parent but absent in the other parent, were tested for their fit to the expected 1:1 (presence:absence) ratio. S_a which was present in both parents was tested for its fit to a 3:1 (presence:absence) ratio.

The most informative markers for linkage mapping from a pseudo-testcross mapping population are single dose restriction fragments (SDRF) that differ between both parents and segregate 1 : 1 (presence : absence) (Wu et al. 1992). Therefore, *S*-alleles which differed between both parents and fit a 1:1 ratio at the 5% level were combined with the existing marker segregation data previously used to construct the 'RS' x 'EB' linkage map (Wang et al. 1998).

Linkage analysis was done with JoinMap V2.0 (Stam 1993) using a minimum LOD score of 3.0. Distances are presented in centi-Morgans calculated by the Kosambi function.

Results

Pollen tube growth studies of sour cherry and sweet cherry interspecific crosses

'Sato Nishiki' (S_3S_6) pollen was able to grow the full length of both 'Crisana' ($S_1S_4S_d$) and 'Tschernokorka' ($S_9S_aS_c$) styles (Table 3.1). 'Rainier' (S_1S_4) pollen was able to grow the full length of the 'Tschernokorka' styles (Table 3.1). However, 'Rainier' pollen tube growth was arrested half way down the 'Crisana' styles (Table 3.1) and swelling was observed at the pollen tube tips. This suggests that the S_1- and S_4-RNases from 'Crisana' were able to recognize and inhibit the S_1- and S_4-pollen from 'Rainier'.

'Tschernokorka' pollen grew the full length of both 'Rainier' and 'Schmidt' (S_2S_4) styles (Table 3.2). 'Crisana' pollen was also able to grow the full length of 'Schmidt' styles; however, 'Crisana' pollen was arrested halfway down the 'Rainier' styles (Table 3.2). This suggests that the S_1- and S_4-RNases from 'Rainier' were able to recognize and degrade the 2x pollen tubes from 'Crisana' that would either be heteroallelic or homoallelic for the S_1-, S_4- and/or S_d-pollen S-alleles.

cDNA cloning from 'RS'

3' RACE cDNA clones encoding four *S*-RNases that had previously been determined to be in 'RS', S_a, S_b, S_c and S_6, were cloned and sequenced. Two of the three unique *S-RNases* from 'EB', S_a and S_4, had previously been cloned and sequenced (Yamane et al. 2001). A cDNA for the S_{6m}, the third *S*-allele postulated to be in 'EB', could not be identified (Yamane et al. 2001). However, this allele was suspected to be functionally similar to the S_6-allele due to complete identity between the S_6 sweet cherry

Table 3.1 Cross-compatibility results for pollination of sour cherry styles with sweet cherry pollen based on examination of pollen tube growth in styles 72 hours after pollination

Style parent	Pollen parent (*S*-allele genotype)	
(*S-RNases*)[a]	'Rainier' (S_1S_4)	'Sato Nishiki' (S_3S_6)
'Crisana' ($S_1S_4S_d$)	Incompatible	Compatible
'Tschernokorka' ($S_9S_aS_c$)	Compatible	Compatible

[a] Three different *S-RNases* have been identified in each parent. At this time, it is not known which of the three *S-RNases* is present in two copies.

Table 3.2 Cross-compatibility results for pollination of sweet cherry cultivars with sour cherry pollen based on examination of pollen tube growth in styles 72 hours after pollination

Style parent	Pollen parent (*S-RNases*)[a]	
(*S*-allele genotype)	'Crisana' ($S_1S_4S_d$)	'Tschernokorka' ($S_9S_aS_c$)
'Rainier' (S_1S_4)	Incompatible	Compatible
'Schmidt' (S_2S_4)	Compatible	Compatible

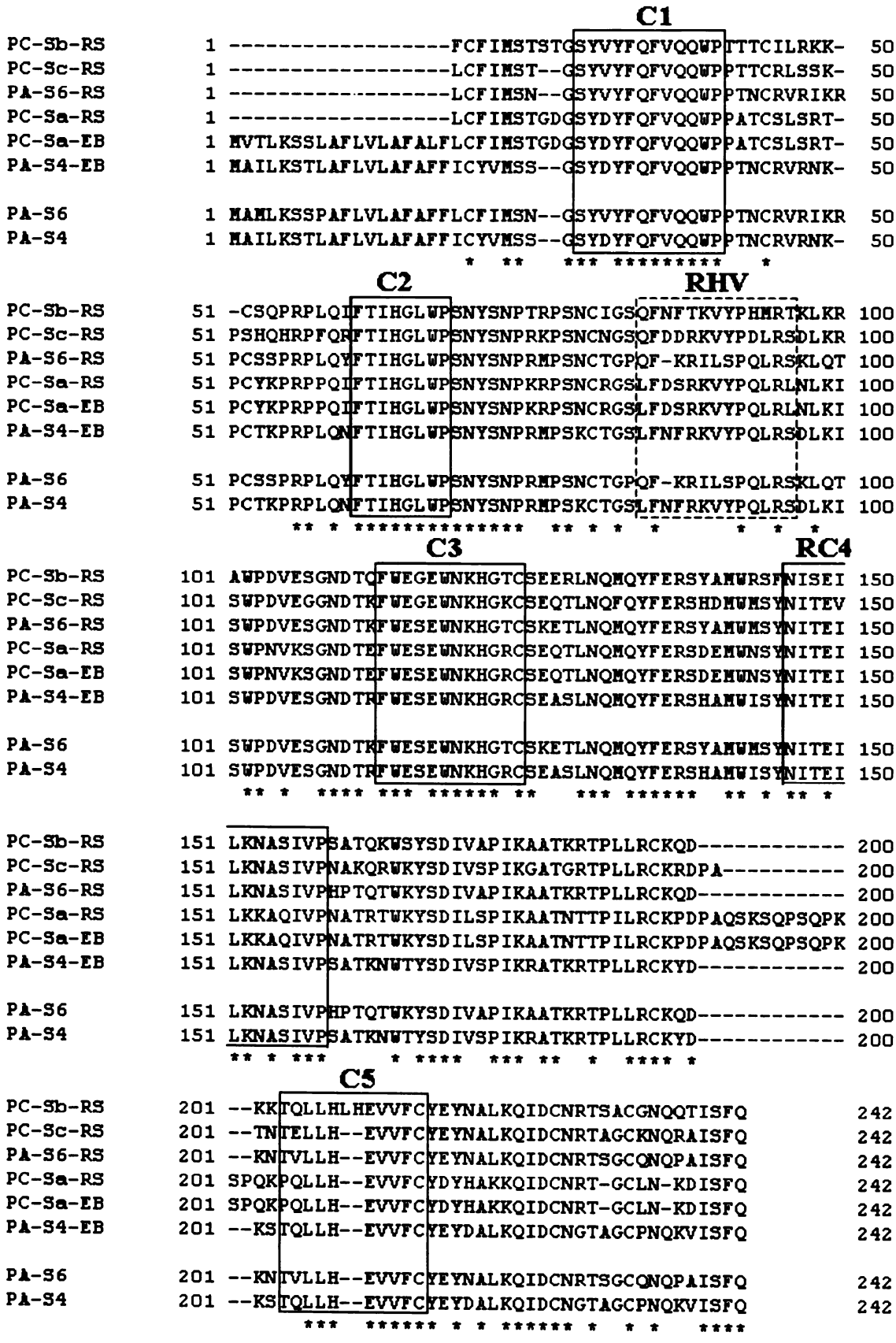
[a] Three different *S-RNases* have been identified in each parent. At this time, it is not known which of the three *S-RNases* is present in two copies

sequence and the sequence amplified from 'EB' using the Pru-C2 and PCE-R primers that span the two hyper-variable regions present in the *Prunus S-RNases* (Tao et al. 1999; Yamane et al. 2001). The amino acid alignment of the four *S*-RNases from 'RS' with the two *S*-RNases from 'EB' is presented along with the amino acid sequences of the sweet cherry S_4 and S_6 sequences (Genbank sequence accessions AB028154 and AB010305, respectively) (Figure 3.1). The partial amino acid sequence of the S_a-RNase from 'RS' was identical to the sequence of the S_a-RNase from 'EB' determined previously (Yamane et al., 2001) (Figure 3.1). The deduced amino acid sequences from the S_b- and S_c-cDNA clones contained the two active domains shared by other T2/S-RNases and the five regions that are conserved among rosaceous *S*-RNases. In addition, seven cysteine residues and an N-glycosylation site conserved among other rosaceous *S*-RNases were present in the amino acid sequences of the S_b- and S_c-RNases. However, the sequences of the cDNAs encoding the S_b- and S_c-*RNases* were not identical to the DNA sequences of any known *S-RNases*, suggesting that they encode novel *S-RNases*. The novel S_b- and S_c-RNases share 63 to 80 % identity with other sweet cherry *S*-RNases, within the range of amino acid sequence identity observed among *Prunus S*-RNases. These results indicated that four different kinds of *S*-RNases, corresponding to the four *S*-alleles in the genome of 'RS', are present in the style of 'RS'.

Inheritance and linkage analysis of the *S*-RNases in the 'RS' x 'EB' mapping population

The *S-RNase* RFLP profiles following *Hin*dIII digestion for 'RS' and 'EB' agreed with the previous report (Yamane et al. 2001). 'RS' exhibited four fragments of 6.4 kb, 5.8 kb, 5.1 kb, and 4.6 kb which correspond to the *S-RNases*, S_a, S_6, S_b and S_c, respectively (Figure 3.2). The four fragments exhibited by 'EB' correspond to the three *S-RNases* S_{6m} (9 kb), S_a (6.4 kb) and S_4 (6.1 kb and 5.6 kb) (Figure 3.2). Partial genomic sequences of the 'RS' and 'EB' derived S_6- and S_{6m}-*RNases*, respectively, were identical

Figure 3.1 Amino acid sequence alignment of four *S*-RNases from 'RS', S_a, S_b, S_c and S_6, two from 'EB', S_a and S_4, (Yamane et al. 2001) and the sweet cherry S_4- and S_6-RNases. The alignment was generated by CLUSTAL X (Thompson et al. 1997). Gaps are marked by dashes. 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 rosaceous *S*-RNases is marked with a dotted box. Conserved amino acid residues are designated by asterisks under the sequences.


C1
C2
RHV
C3
RC4
C5
PC-Sb-RS
PC-Sc-RS
PA-S6-RS
PC-Sa-RS
PC-Sa-EB
PA-S4-EB
PA-S6
PA-S4

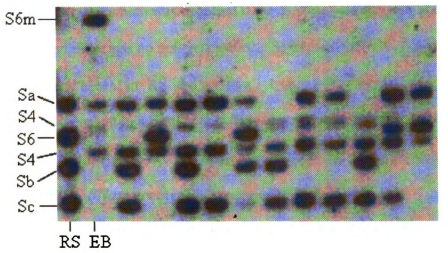


Figure 3.2 Genomic blot analysis of 'RS', 'EB' and eleven progeny. Six micrograms of genomic DNA were digested by *Hin*dIII and hybridized to the cDNA encoding the S_6-*RNase*.

suggesting that they are the same *S-RNases* and that the RFLP polymorphism occurs outside the *S-RNase* coding region (Yamane et al. 2001).

S-RNase segregation in 85 progeny from the 'RS' x 'EB' mapping population was determined (Table 3.3). Segregation of the *S*-alleles that were present in the maternal parent, 'RS', but absent in the paternal parent, 'EB', (S_b, S_c, and S_6) fit the expected 1:1 ratio (Table 3.3, Figure 3.2). Segregation of the S_a-allele, which was present in both 'RS' and 'EB', fit a 3:1 ratio, which was expected if the *S-RNase* was present in only one dose in each parent and there was no pollen selection. Segregation for the S_4-allele which was present in 'EB' and absent in 'RS' did not fit a 1:1 ratio which would be expected if the S_4-allele was only present in a single dose. However, segregation of S_4 in 'EB' fit a 5:1 ratio suggesting that there are two S_4-alleles exhibiting tetrasomic inheritance and therefore the 'EB' *S-RNase* genotype is presumed to be $S_{6m}S_4S_4S_a$. The S_{6m} in 'EB' that could be distinguished from the S_6-allele in 'RS' by RFLP analysis following *Hin*dIII digest (Figure 3.2) was not present in any of the progeny.

The three *S-RNase* alleles that fit a 1:1 segregation ratio, S_b, S_c and S_6, all from the maternal parent, RS, were used for linkage analysis. The S_b-allele mapped to 'RS' linkage group 6 of the framework map constructed by Wang et al. (1998) (Figure 3.3). The 'RS' linkage group 6 consisted of 17 markers spanning 34.4 cM and the S_b-locus mapped 4.5 cM from the marker placed at one end of this linkage group. The other two *S*-alleles, S_6 and S_c, were linked to each other at a distance of 23.2 cM and unlinked to any other previously identified markers. When the progeny SI and SC phenotypes were entered as data in the linkage map analysis, the SI and SC phenotypes did not segregate

Table 3.3 RFLP segregation of *S-RNase* alleles in the 'RS' x 'EB' mapping population [z]

S-allele	RS	EB	Expected ratio	Presence: absence ratio in progeny [y]	χ^2 value
S_a	+	+	3 :1	69 : 15	2.29
S_b	+	-	1 :1	34 : 51	3.4
S_c	+	-	1:1	46 : 37	0.98
S_4	-	+	1:1	77 : 8	56.0 **
			5:1[x]		3.25
S_6	+	-	1:1	45 : 40	0.29
S_{6m}	-	+	1:1	0 : 85	85.0 **

** denotes significance at the 0.001 level

[z] The *S*-genotypes of 'RS' and 'EB' were provisionally determined to be $S_aS_bS_cS_6$ and $S_aS_4S_{6m}$, respectively, by Yamane et al. (2001)

[y] Only the data for those progeny that could be unambiguously scored for each *S*-allele was included

[x] 5:1 is the expected segregation ratio for an allele that is present in two copies which exhibits tetrasomic inheritance

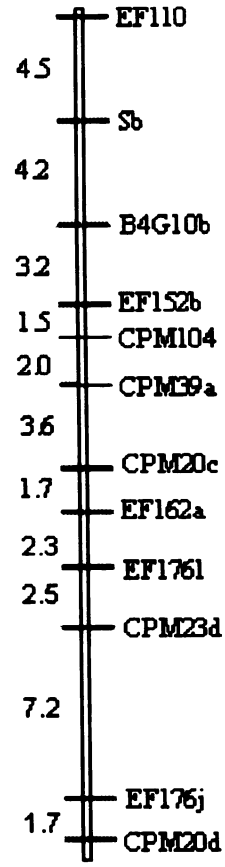


Figure 3.3 Genetic map for the sour cherry linkage group 6 obtained with the 'RS' x 'EB' mapping population showing the location of the *S_b-RNases* (Sb). The framework map was created by Wang et al. (1998). Markers shown on the right are identified by the probe followed by a letter (i.e., *a*, *b*, *c*, etc.) when more than one marker is generated from a single probe.

with any markers suggesting that SI and SC in sour cherry is controlled by segregation at more than one locus.

SI and SC phenotypes of progeny from the 'RS' x 'EB' mapping population

Both self- and outcross-pollen grew the full length of 'RS' and 'EB' styles during pollen tube growth studies, confirming that both cultivars are SC. The SI or SC phenotype for 65 of the 85 individuals in the mapping population could also be determined. Thirty-nine progeny were determined to be SC, while 26 were SI. Nine other trees had died before their phenotype could be determined. Six trees produced no functional pollen during either of the two pollination seasons, and were thus concluded to be male sterile. Low fertility among the remaining six progeny caused ambiguous SI or SC phenotype results, thus no phenotype could be assigned.

When the SI and SC phenotypes were compared with the RFLP *S-RNase* patterns detected for the 65 progeny tested for both traits, the presence/absence of one or more *S-RNase* fragment(s) was not associated with either SI or SC. In addition, certain *S*-allele genotypes segregated for SI and SC phenotype.

Discussion

Inter-specific crosses between sweet and sour cherry demonstrate that gametophytic self-incompatibility exists in sour cherry and that the recognition of common *S*-alleles has been maintained in spite of polyploidization. The results from the reciprocal crosses of

'Crisana' ($S_1S_4S_d$) and 'Rainier' (S_1S_4) indicate that the S_1- and S_4-stylar and pollen components in the two selections are functionally similar.

The incompatible reaction in the cross 'Crisana' ($S_1S_4S_d$) x 'Rainier' (S_1S_4) can be explained by the recognition of the 'Rainier' S_1- and S_4-pollen *S*-genes by the 'Crisana' S_1- and S_4-RNases, respectively. However, an explanation for the incompatibility of the reciprocal cross, 'Rainier' x 'Crisana', is less straightforward. Since it is expected that like most sour cherry cultivars, 'Crisana' exhibits occasional inter-genomic pairing (Murawski and Endlich 1962) six types of pollen would be possible: heteroallelic (i.e. S_1S_4, S_1S_d, S_4S_d) and homoallelic (S_1S_1, S_4S_4, S_dS_d) depending on which allele is in double dose. The relative frequencies of these pollen types would also be dependent on dosage and pairing configuration. However, without confirming the precise pollen genotypes and the functional activity of the different *S*-haplotypes it is premature to speculate on the mechanism of pollen recognition and inhibition.

The results from the cross 'RS' x 'EB' suggest that only one *S*-allele match between the style and pollen is necessary to render diploid pollen incompatible. In the cross, the genotypes of 'RS' and 'EB' were determined to be $S_aS_bS_cS_6$ and $S_aS_4S_4S_{6m}$, respectively. In the progeny, it was possible to follow the inheritance of the 'RS'-derived S_6-allele and the 'EB'-derived S_{6m}-allele because these two alleles could be differentiated by RFLP analysis following *Hin*dIII digestion. Since 'EB' only has one S_{6m}-allele, all the pollen containing S_{6m} are expected to be heteroallelic. However, in this cross no progeny individuals contained the 'EB'-derived S_{6m}-allele. This observation is in contrast to results from several Solanaceous species where heteroallelic pollen loses its SI phenotype (Livermore and Johnstone 1940; Stout and Chandler 1942; Crane and Lewis 1942;

Brewbaker 1954; Pandy 1968; de Nettancourt et al. 1974; Chawla et al. 1997; Entani et al. 1999; Golz et al. 1999; Luu et al. 2001)

The previously reported inability to identify any S_6-RNase associated protein or cDNA in 'EB' (Yamane et al. 2001) suggests that the S_{6m}-RNase is not functionally active in the 'EB' style. The segregation data suggests however, that the 'EB' derived S_{6m}-pollen component is functional and is inhibited by the 'RS' S_6-RNase. It would therefore be postulated that in the reciprocal cross, 'EB' x 'RS', both the 'RS'- and 'EB'-derived S_6-alleles should be inherited by a portion of the progeny.

Given the putative *S*-genotype of 'EB' ($S_aS_4S_4S_{6m}$) and the inability of pollen containing the S_{6m}-allele to successfully fertilize 'RS', all progeny should inherit at least one copy of the S_4-allele from the 'EB' parent. However, eight of the 85 progeny that were genotyped for their *S*-RNase alleles did not contain an S_4-RNase. One possible explanation is that the S_{6m}-allele region of 'EB' that contains the pollen *S* and *S*-RNase genes has been translocated leaving an *S*-null allele. Therefore, these eight progeny that do not contain an S_4-RNase would have obtained S_a- and *S*-null alleles from the 'EB' parent. These plants consisted of both SC and SI types.

Both linkage mapping parents, 'RS' and 'EB', have one S_a-*RNase* allele. Progeny segregation for this allele (3:1, present:absent) suggested that both parental copies of the S_a-allele were inherited by the progeny. This result would be expected if heteroallelic pollen from 'EB' carrying the S_a-allele and another *S*-allele were SC. However, data from the progeny did not support this hypothesis. Sixteen progeny were obtained that, like both parents, contained only one copy of the S_a-allele. Since five of these 16 progeny were SI, heteroallelic pollen with just one S_a is not sufficient by itself to cause

the plant to be SC. In the sour cherry cross, the ability of pollen with an S_a-allele to grow down a style containing an S_a-RNase may be possible if at least one of the S_a-alleles is non-functional due to a mutation in either the *S-RNase* or *pollen S*-gene coupled with the presence of a second non-functional *S*-allele in either the style or pollen.

Whereas the current study provided additional evidence of *S-RNase* segregation in sour cherry, the actual cause of SC versus SI in sour cherry was not determined. For example, no *S-RNase*(s) have been found to co-segregate with SC in the 'RS' x 'EB' population, as has been found in some SC mutants (Tao et al. 2000), so it is unlikely that a single *S-RNase* mutation is responsible for causing self-compatibility. One possible mechanism for the existence of SC in sour cherry is the existence of mutations in the *S*-RNase or pollen *S*-gene of a number of *S*-alleles. Therefore the identification of the pollen *S*-gene(s) in sour cherry is likely to be crucial to the understanding of SC and SI in sour cherry. In addition, certain progeny with similar *S*-haplotypes differed with respect to their SI or SC phenotypes. This suggests that modifier genes may have a role in modulating the interaction between the *S*-pollen protein and the *S*-RNase. Modifier genes have been demonstrated to be required for normal SI function in *Nicotiana* (McClure et al. 1999).

Along with the crossing studies described above, amino acid matches of the sweet cherry and sour cherry S_4- and S_6-alleles, indicate that the alleles that were suspected to be in common between these two species are identical. This is additional evidence that gametophytic SI occurs in sour cherry and that it is regulated by a similar mechanism as found in other gametophytic SI species in which a stylar *S*-RNase interacts with a pollen-specific molecule to regulate the SI or SC response.

Since sour cherry is an allotetraploid, it is likely that the two *S*-loci would map to homoeologous linkage groups. The *S_b-RNase* mapped to the expected position on *Prunus* linkage group 6 indicating that this linkage relationship is maintained among sour cherry, sweet cherry and almond. The two other S-*RNases*, S_c and S_6, mapped together on what might represent a new homoeologous linkage group 6 that had previously been undetected by Wang et al. (1998) due to low marker density on the sour cherry map. However, S_c and S_6 would have been expected to map to the same location and it is not clear why they are separated by over 20 cM. Selection for or against certain *S*-alleles or other alleles in the *S*-locus region could have occurred. In addition meiotic irregularities prevalent in sour cherry can complicate linkage analysis. Although sour cherry is an allotetraploid predominately exhibiting disomic inheritance, it also exhibits tetrasomic inheritance (Beaver et al. 1993) and quadrivalent pairing characteristic of an autotetraploid (Wang 1998). For example, all twenty of the 'EB' metaphase I pollen mother cells (PMC) examined had some non-bivalent pairing with at least one quadrivalent per PMC. Therefore the tetrasomic inheritance exhibited by the 'EB' S_4-alleles is not unexpected. However, it is also possible that the S_c –S_6 linkage result is caused by an actual change in the physical location of the *S*-locus. This is not unprecedented since a translocation has been confirmed in almond between *Prunus* linkage groups 4 and 6 (P. Arus, pers. comm.).

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CHAPTER 4

GENETIC CONTROL OF SELF-INCOMPATIBILITY AND SELF-COMPATIBILITY IN TETRAPLOID SOUR CHERRY (*PRUNUS CERASUS* L.)

Abstract

Gametophytic self-incompatibility (SI) in diploid species generally breaks down due to polyploidy, resulting in self-compatible (SC) tetraploid species. The diploid sweet cherry and the tetraploid sour cherry represent an exception, as sour cherry individuals can be either SI or SC. To investigate the genetic control of SI and SC in sour cherry, the segregation of *S*-haplotypes in a total of 794 progeny from six sour cherry self-populations and 15 inter-specific crosses between sweet and sour cherry involving six SC sour cherry selections were analyzed. Results indicate that the breakdown of SI in *Prunus* is caused by the accumulation of non-functional *S*-haplotypes that are incapable of *S*-haplotype-specific rejection of pollen, rather than due to the competition between pollen-*S* products in heteroallelic pollen, as commonly observed in the Solanaceae. In total, four fully functional *S*-haplotypes and six non-functional *S*-haplotypes were discovered in these six sour cherry selections. A hypothesis regarding the genetic control of SI and SC in sour cherry was developed and verified in self-pollination and crossing experiments. The implications of these findings on sour cherry breeding and on our knowledge of gametophytic SI are discussed.

Introduction

Gametophytic self-incompatibility (GSI) is a common mechanism for promoting out-crossing in flowering plants (de Nettancourt, 1977). In GSI, self-incompatibility (SI) is determined by a single, multi-allelic locus, called the *S*-locus, in which the compatibility of a cross is determined by the haploid genome of the pollen and the diploid genome of the pistil. Pollen tube growth is arrested if the pollen tube has an *S*-allele in common with one of the two *S*-alleles in the style. The *S*-locus contains a minimum of two genes, one controlling the style-specificity and the other the pollen-specificity of the SI reaction. The stylar-determinant is an RNase (*S-RNase*) (Anderson, 1986; McClure et al., 1989), which is specifically expressed in the pistil and specifically degrades rRNA of incompatible pollen (McClure et al., 1990). The identity of the *pollen-S* gene remains unconfirmed; however, *S*-locus linked F-box genes have been proposed to be the *pollen-S* gene in *Anthirrimum* (*SLF*; Lai et al., 2002), *Prunus mume* (*SLF*; Entani et al. 2003) and *P. dulcis* (*SFB*; Ushijima et al. 2003). The inability to transform these species precludes the necessary transformation experiments to verify that one of these F-box genes is the *pollen-S* gene. Transformation experiments involving a recently discovered *S*-locus F-box gene in *Petunia inflata* (*PiSLF*) suggests that it is the *pollen-S* gene in the Solanaceae (Sijacic et al., 2004).

The *S-RNase* gene from the Solanaceae and the Rosaceae are thought to be orthologous (Igic and Kohn, 2001). However, several differences between *Prunus* and other Rosaceous and Solanaceous species have been reported. The *S-RNase* gene of *Prunus* contains two introns, whereas the *S-RNase* for all other Rosaceous and

Solanaceous species contains only one (Igic and Kohn, 2001). Second, the putative *pollen-S* F-box genes isolated from *Antirrhinum* (Lai et al., 2002) and *Prunus* (Entani et al., 2003; Ushijima et al., 2003) do not appear to be orthologous. Third, screens for pollen-part mutants (PPMs) in the Solanaceae have failed to reveal a PPM caused by the mutation of the *pollen-S* gene (Pandey 1967; van Gastel and de Nettancourt, 1975; Golz et al., 1999; Golz et al., 2000). Instead, all PPMs have included duplications of the *pollen-S* gene, suggesting that a deletion or knock out of the *pollen-S* gene is lethal. However, two PPMs, S_4' from sweet cherry and S_f from *P. mume*, that were caused by the knock out of *pollen-S* function have been characterized in *Prunus* (Ushijima et al., 2004). Together, these findings suggest that the identity and/or function of the *pollen-S* gene may differ in *Prunus* and the Solanaceae.

GSI typically breaks down as a result of polyploidy (Crane and Lawrence, 1931; Crane and Thomas, 1939; Livermore and Johnstone, 1940; Stout and Chandler, 1941; Pandey 1968; de Nettancourt et al., 1974). It is hypothesized that the competition between multiple different pollen-*S* products in a single pollen grain eliminates the ability of the pollen to initiate an SI reaction, allowing these heteroallelic pollen grains to successfully grow through styles despite the presence of their cognate *S*-RNases (Stout and Chandler, 1942; Lewis 1943; Chawla et al., 1997; Entani et al., 1999). However, a recent comparison of ploidy level and the presence of self-compatibility (SC) among angiosperms indicates that there is no association between ploidy level and SC (Mable 2004). The frequency of SI and SC species was not significantly different for diploids and tetraploid species.

Sweet cherry (*P. avium*) is a diploid species that has a classical RNase-based GSI system (Bošković and Tobutt, 1996) with 17 identified *S*-haplotypes (Mathews and Dow, 1969; Tao et al., 1999; Bošković and Tobutt, 2001; Hauck et al., 2001; Sonneveld et al., 2001; Wiersma et al., 2001; Choi et al., 2002; Sonneveld et al., 2003; Iezzoni et al., 200x; Wunsch and Hormaza, 2004). Sweet cherry is one of the progenitor species of the allotetraploid sour cherry (*P. cerasus*). Sour cherry also contains a GSI system characterized by the premature cessation of incompatible pollen tube growth in the styles (Lansari and Iezzoni, 1990; Yamane et al., 2001). In addition, reciprocal inter-specific crosses between sweet and sour cherry indicate that sour cherry has retained its ability to reject pollen in an *S*-haplotype-specific manner (Hauck et al., 2002). However, a partial breakdown of SI has occurred in sour cherry, resulting in the existence of both SC and SI types (Lech and Tylus, 1983; Redalen, 1984; Lansari and Iezzoni, 1990). The genetic cause of the partial breakdown of SI in sour cherry is unknown.

It remains to be seen whether sour cherry pollen that contains two different pollen-*S* products loses its SI phenotype, as in the Solanaceae, or whether a different mechanism causes the observed partial breakdown of SI in sour cherry. A second possible mechanism for the breakdown of SI is that mutations in a modifier gene, such as the *HT* gene from *Nicotiana* (McClure et al., 1999), causes the breakdown of SI in sour cherry, as suggested by progeny from a cross between Rheinische Schattenmorelle (RS) and Érdi Bőtermő (EB) that contain the same *S-RNase* phenotype but segregate for SI and SC (Hauck et al., 2002). A third possibility is that mutations in one or both of the specificity components, *S-RNase* or *pollen-S*, have accumulated and the inability of these non-functional *S*-haplotypes to carry out S-haplotype specific rejection causes the partial

breakdown of SI in sour cherry. One such non-functional sour cherry *S*-haplotype, S_{6m}, has been characterized and found to have a fully functional *pollen-S* gene that was identical to that of the S_6 haplotype of sweet cherry. The *S-RNase* of the S_{6m}-haplotype, however, was non-functional due to the insertion of a 2.6 kb fragment upstream from the S_6-*RNase* gene, which eliminated gene expression (Yamane et al., 2003). A more in depth genetic characterization of *S*-haplotypes is necessary to differentiate between these three possibilities.

The ultimate goal of this research was to determine the genetic control of SI and SC in sour cherry. In this study, systematic genetic analyses of previously defined *S*-haplotypes were conducted using both inter-specific crosses between sweet and sour cherry and sour cherry self-populations. Finally, a hypothesis was developed for the genetic control of SI and SC in sour cherry and verified using self-pollination and crossing experiments. Implications of the findings on the evolution and the effects of polyploidy on GSI are discussed.

Materials and Methods

Plant material

All sweet and sour cherry cultivars, along with their proposed *S*-haplotypes, used in this study are listed in Table 4.1. The sour cherry *S*-haplotype nomenclature proposed by Yamane et al. (2001) is used in this paper, with the S_4, S_6 and S_9-haplotypes representing RFLP profiles identical to *S*-haplotypes in sweet cherry, and S_a, S_b, S_d, and

Table 4.1: Sweet and sour cherry cultivars used in this study, with their proposed *S*-haplotypes

Cultivar	SI or SC	S-haplotype[a]
Sweet Cherry		
Chelan	SI	S_3S_9
Emperor Francis (EF)	SI	S_3S_4
Gold	SI	S_3S_6
Regina	SI	S_1S_3
Schmidt	SI	S_2S_4
Sour Cherry		
Cigány 59	SC	S_6S_9/S_aS_b
Érdi Bőtermő (EB)	SC	S_4S_6/S_aS_x[b]
Montmorency	SC	$S_6S_{13'}/S_aS_x$[b,c]
Rheinische Schattenmorelle (RS)	SC	$S_6S_{13'}/S_aS_b$[c]
Surefire	SC	$S_4S_{13'}/S_aS_x$[b,c]
Újfehértói fürtös (UF)	SC	S_4S_e/S_dS_x[b]

[a] Sour cherry is an allotetraploid. The homologous pairing of *S*-haplotypes are designated. Homologous pairing was determined based on observed segregation of *S*-haplotypes in this study.

[b] In those sour cherry cultivars where only three *S*-haplotypes could be identified, S_x is used to denote either a null allele or the double dosage of one of the other *S*-haplotypes.

[c] $S_{13'}$ was previously named S_c (Yamane et al., 2001). Alignment of the predicted amino acid sequence of the S_{13}-RNase from sweet cherry (GenBank accession number: AJ635276) and S_c from sour cherry (Hauck et al., 2002) indicates that these two *S-RNases* are identical.

S_e having unique RFLP banding profiles. The previously reported S_c has been renamed $S_{13'}$ due to identical amino acid sequences of the sweet cherry S_{13}-RNase (GenBank accession number: AJ635276) and the S_c-RNase (Hauck et al., 2002). The "" indicates a hypothesized pollen-part mutation. The trees are located either at the Michigan State University Clarksville Horticultural Experimental Station (CHES), Clarksville, Mich. or the MSU Northwest Research Station (NWRS), Traverse City, Mich.

Field crosses and self-pollinations

The inter-specific crosses and self-pollinations that were analyzed in this study are listed in Tables 4.2 and 4.3, respectively. Anthers were collected from flowers in the late balloon stage, allowed to dry at room temperature overnight, and either used immediately or stored in glass vials at -20°C with a desiccant. One day prior to bloom, about 200 flowers per cross were emasculated. The next morning, the dried anthers were crushed with glass rods and applied directly to the stigmas. Immediately following fruit ripening, the seed were harvested, cleaned from the surrounding fruit, and stored at -20°C until used for DNA isolation.

DNA isolation

DNA isolation from leaves of parents: Young, unfolded leaves of fully-grown trees were placed on dry ice immediately following collection, stored at -80°C overnight and then lyophilized for 48 h. DNA isolation was performed using the CTAB method described by Stockinger et al. (1996).

DNA isolation from seed: Seed from the crosses were harvested shortly after ripening and stored at -20°C. The testa was removed to allow DNA extraction from the embryo and cotyledons. The embryo and cotyledons were crushed using liquid nitrogen and

Table 4.2: Reciprocal inter-specific crosses between sour cherry and sweet cherry used to investigate the functionality of the S_4, S_6 and S_9-haplotypes from sour cherry.

Parents used in cross		S-haplotype	No. of progeny analyzed	
Sour cherry	Sweet cherry	tested	sour x sweet	sweet x sour
Cigány 59 ($S_{6c}S_9S_aS_b$)	Gold (S_3S_6)	S_6	36	40
Cigány 59 ($S_{6c}S_9S_aS_b$)	Chelan (S_3S_9)	S_9	7	0 [a]
EB ($S_4S_{6m}S_aS_x$)	EF (S_3S_4)	S_4	10	20
EB ($S_4S_{6m}S_aS_x$)	Gold (S_3S_6)	S_6	33	14
Montmorency ($S_6S_{13'}S_aS_x$)	Gold (S_3S_6)	S_6	55	15
RS ($S_6S_{13'}S_aS_b$)	Gold (S_3S_6)	S_6	31	13
Surefire ($S_4S_{13'}S_aS_x$)	EF (S_3S_4)	S_4	30	37
UF ($S_4S_dS_eS_x$)	Schmidt (S_2S_4)	S_4	45	0 [b]
UF ($S_4S_dS_eS_x$)	EF (S_3S_4)	S_4	0 [b]	36

[a] Chelan was not used as the maternal parent. Thus, the functionality of the *pollen-S_9* gene from Cigány 59 could not be tested.

[b] Different sweet cherry testers were used for the reciprocal crosses with UF.

Table 4.3: Sour cherry self-populations analyzed to determine the functionality of the $S_{13'}$, S_a, S_b, S_d and S_e-haplotypes.

Cultivar self-pollinated	No. of progeny analyzed
Cigány 59	37
Érdi Bőtermő	8
Montmorency	135
Rheinische Schattenmorelle	54
Surefire	36
Újfehértói fürtös	102

mixed in a buffer consisting of 150 mM Tris-HCl (pH 8.0), 20 mM EDTA, 800 mM NaCl, 0.25% SDS, 1% β-mercaptoethanol and 1% CTAB. The DNA was purified by two chloroform extractions and precipitated using isopropanol. See Appendix A for complete protocol.

PCR amplification of *S-RNases*

The primer combination, Pru-C2 and PCE-R, which hybridized to conserved regions flanking the second highly variable intron of the *S-RNase* gene, were initially used to amplify the sweet and sour cherry *S*-haplotypes simultaneously (Yamane et al., 2001). This primer set did not reliably amplify S_2 or $S_{13'}$, so *S*-allele specific primers were used to amplify these *S*-haplotypes (S_2: Sonneveld et al., 2001; S_{13}: Sonneveld et al., 2003). Pru-C2 and PCE-R also did not distinguish between S_9 and S_b, so *S*-haplotype specific primers were used for crosses that contain both of these *S*-haplotypes (S_9: Sonneveld et al., 2003). S_b-specific primers did not exist and were designed from previously obtained cDNA sequences (Hauck et al., 2002). Table 4.4 shows the sequences and annealing temperatures for all PCR primers used in this study. A similar PCR temperature profile, other than the annealing temperature, was used for all PCR reactions: an initial denaturing step (94°C, 2.5 min) followed by 35 cycles of 94°C (30 sec), X°C (30 sec), 72°C, (90 sec) and a final elongation step (72°C, 5 min).

***S*-genotype or S-phenotype determination for hypothesis testing**

Six micrograms of DNA from 93 sour cherry selections chosen for SI or SC evaluation was digested with *Hin*dIII (Boehringer Mannheim Biochemicals,

Table 4.4: DNA sequences, annealing temperature, and references for *S-RNase* genotyping PCR primers used in this study.

Primer	Sequence (5'-3')	Annealing Temp. (°C)	Reference[a]
PruC2	CTA TGG CCA AGT AAT TAT TCA AAC C	56	T
PCE-R	TGT TTG TTC CAT TCG CYT TCC C		Y
PaS2-F	TAC TTC GAG CGA TCC CAA A	50	S
PaS2-R	AAG TGC AAT CGT TCA TTT G		S
PaS9-F	TT TGT TAC GTT ATG AGC AGC AG	62	R
PaS9-R	ATG AAA CAA TAC ATA CCA CTT TGC TA		R
PaS13-F	CA ATG GGT CGC AAT TTG ACG A	66	R
PaS13-R	GGA GGA GGT GGA TTC GAA CAC TTG		R
PcSb-F	CAC CTG CAT ACT TCG CAA GA	66	
PcSb-R	TGC TGC TTT AAT GGG TGC TA		

[a] T (Tao et al., 1999); Y (Yamane et al., 2001); S (Sonneveld et al., 2001); R (Sonneveld et al., 2003)

Indianapolis), resolved on a 0.9 % agarose gel for 36 h at 30 V, and transferred to a nylon membrane (Hybond-N+, Amersham) according to Wang et al. (1998). PCR amplified fragments of the S_6-RNase cDNA from sweet cherry (Tao et al., 1999) were used as the probe. This probe has been shown to cross-hybridize with all sweet and sour cherry *S-RNases* (Tao et al., 1999; Yamane et al., 2001; Hauck et al., 2002). Probes were radiolabelled with ^{32}P-dCTP (DuPont, Boston) using the random primer hexamer-priming method described by Feinberg and Vogelstein (1983). After hybridization at 60°C for 16 hours and high stringency washes (2 × 30 min with 2 X SSC and 1 % SDS followed by 2 × 30 min with 0.2 X SSC and 0.5 % SDS at 60 °C), radioactive signal was detected on X-ray films using a Kodak RP X-OMAT processor.

Analysis of pollen tube growth for hypothesis testing

The 93 sour cherry selections were tested for SI or SC by observing pollen tube growth in self-pollinated styles. Pollination tests were performed as described by Lansari and Iezzoni (1990) with slight modifications. Pollen from newly opened flowers was collected and dried for at least 24 h. Twenty flowers at the balloon stage were emasculated. Ten emasculated flowers were hand pollinated with self-pollen when receptive (24 h after emasculation) and ten flowers were pollinated with a mixture of pollen from ten random sour cherry selections ("bulk pollen"). The pollinated pistils were collected 72 h after pollination, placed in fixing solution [(1 chloroform: 3 (95%) ethanol: 1 glacial acetic acid) (v/v)] for 24 h, transferred into 100% ethanol, and stored at 4 °C until used for observation. The pistils were washed thoroughly in tap water, incubated in 10 N NaOH for 4 h to soften the tissues, and soaked in 0.1 % aniline blue solution with

33 mM K_3PO_4 (pH 8.5) for 45 min to stain the pollen tubes. Pollen tubes were observed by ultraviolet fluorescent microscopy (BX60, Olympus, Tokyo, Japan).

Results

Inter-specific crosses

Reciprocal inter-specific crosses between sweet and sour cherry were designed to take advantage of their shared *S*-haplotypes to systematically test the functionality of the sour cherry S_4, S_6 and S_9-haplotypes. The sweet cherry testers were Schmidt or Emperor Francis (EF) (S_4), Gold (S_6) and Chelan (S_9). The segregation of every *S*-haplotype in each of the inter-specific populations is summarized in Table 4.5. The segregation data that is most critical for dissecting the genetic control of SI in sour cherry is described below. Schematic representations of these crosses can be found in the Appendix (Figure B.2).

Surefire x Emperor Francis (EF): All 30 progeny contained the S_3-haplotype; thus all progeny resulted from fertilization of Surefire by EF pollen that contained the S_3-haplotype. This indicates that pollen containing the S_4 pollen-*S* products were recognized and destroyed in a *S*-haplotype-specific manner by a functional S_4-RNase in Surefire styles.

Table 4.5: Segregation of *S*-haplotypes and *S*-genotypes/phenotypes[a] in triploid progeny from sweet cherry x sour cherry reciprocal crosses to test the functionality of the S_4, S_6 and S_9-haplotypes in sour cherry.

Surefire (S_4 $S_{13'}$$S_a$$S_x$[b]) x Emperor Francis ($S_3S_4$)

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	30:0	28.0	<0.0001	S_3	30	$S_3S_aS_{13'}$ (10); $S_3S_{13'}S_x$ (8); $S_3S_4S_a$ (7); $S_3S_4S_x$ (4); $S_3S_aS_x$ (1)
	1:0	30:0	-	-			
S_4	1:1	11:19	1.63	0.20	S_4	0	-
	3:1	11:19	21.5	<0.0001			
$S_{13'}$	1:1	18:12	0.83	0.36			
S_a	1:1	18:12	0.83	0.36			

Emperor Francis (S_3S_4) x Surefire (S_4 $S_{13'}$$S_a$$S_x$[b])

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	22:15	0.97	0.32	$S_{13'}S_x$	19	$S_3S_{13'}S_x$ (11); $S_4S_{13'}S_x$ (8)
S_4	1:1	15:22	0.97	0.32	$S_aS_{13'}$	15	$S_3S_aS_{13'}$ (8); $S_4S_aS_{13'}$ (7)
	3:1	15:22	21.6	<0.0001	S_aS_x[e]	3	$S_3S_aS_x$ (3)
$S_{13'}$	1:1	34:3	24.3	<0.0001	S_4S_a	0	-
S_a	1:1	18:19	0.00	1.00	$S_4S_{13'}$[e]	0	-
					S_4S_x	0	-

Újfehértói fürtös ($S_4S_aS_eS_x$[b]) x Schmidt (S_2S_4)

	S-haplotype segregation				Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_2	1:1	45:0	43.0	<0.0001	S_2	45	$S_2S_eS_x$ (12); $S_2S_4S_x$ (10); $S_2S_aS_e$ (10);
	1:0	45:0	-	-			$S_2S_aS_x$ (5); $S_2S_4S_d$ (5); $S_2S_4S_e$ (3)
S_4	1:1	18:27	1.42	0.23	S_4	0	-
	3:1	18:27	27.6	<0.0001			
S_d	1:1	20:25	0.36	0.55			
S_e	1:1	25:20	0.36	0.55			

Emperor Francis (S_3S_4) x Újfehértói fürtös ($S_4S_aS_eS_x$[b])

	S-haplotype segregation				Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	14:22	1.36	0.24	S_eS_x	20	$S_4S_eS_x$ (10); $S_3S_eS_x$ (10)
S_4	1:1	22:14	1.36	0.24	S_aS_e	14	$S_4S_aS_e$ (11); $S_3S_aS_e$ (3)
	3:1	22:14	3.00	0.08	S_aS_x[e]	2	$S_4S_aS_x$ (1); $S_3S_aS_x$ (1)
S_d	1:1	16:20	0.25	0.62	S_4S_d	0	-
S_e	1:1	34:2	26.7	<0.0001	S_4S_e[e]	0	-
					S_4S_x	0	-

Erdi Botermo ($S_4S_{6m}S_aS_x$[b]) x Emperor Francis (S_3S_4)

	S-haplotype segregation				Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	10:0	8.1	0.004	S_3	10	$S_3S_{6m}S_a$ (4); $S_3S_4S_a$ (3);
	1:0	10:0	-	-			$S_3S_{6m}S_x$ (2); $S_3S_4S_x$ (1)

(Continued)

S_4	1:1	4:6	0.10	0.75	S_4	0	-
	3:1	4:6	4.80	0.03			
S_{6m}	1:1	6:4	0.10	0.75			
S_a	1:1	7:3	0.90	0.34			

Emperor Francis (S_3S_4) x Erdi Botermo ($S_4S_{6m}S_aS_x$[b])

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	10:10	0.05	0.82	$S_{6m}S_x$	11	$S_3S_{6m}S_x$ (7); $S_4S_{6m}S_x$ (4)
S_4	1:1	10:10	0.05	0.82	$S_{6m}S_a$	7	$S_4S_{6m}S_a$ (5); $S_3S_{6m}S_a$ (2)
	3:1	10:10	0.05	0.82	S_aS_x[e]	2	$S_3S_aS_x$ (1); $S_4S_aS_x$ (1)
S_{6m}	1:1	18:2	11.25	0.0008	S_4S_{6m}[e]	0	-
S_a	1:1	9:11	0.05	0.82	S_4S_a	0	-
					S_4S_x	0	-

Erdi Botermo ($S_4S_{6m}S_aS_x$[b]) x Gold (S_3S_6)

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	22:11	3.03	0.08	S_3	22	$S_3S_4S_x$ (6); $S_3S_6S_a$ (6); $S_3S_6S_x$ (4);
S_6 / S_{6m}	1:1	24:9	5.94	0.02			$S_3S_6S_4$ (3); $S_3S_4S_a$ (3)
	3:1	24:9	0.01	0.92	S_6	11	$S_6S_4S_x$ (4); $S_6S_aS_x$ (3); $S_6S_xS_x$ (2);
S_4	1:1	18:15	0.12	0.73			$S_6S_4S_a$ (2)
S_a	1:1	14:19	0.48	0.49			

(Continued)

Gold (S_3S_6) x Erdi Botermo ($S_4S_{6m}S_aS_x$[b])

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square [d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	8:6	0.07	0.79	S_4S_a	7	$S_3S_4S_a$ (5); $S_6S_4S_a$ (2)
S_6 / S_{6m}	1:1	6:8	0.07	0.79	S_4S_x	5	$S_6S_4S_x$ (4); $S_3S_4S_x$ (1)
	3:1	6:8	6.09	0.01	S_aS_x[e]	2	$S_3S_aS_x$ (2)
S_4	1:1	12:2	5.79	0.02	S_4S_6[e]	0	-
S_a	1:1	9:5	0.64	0.42	S_6S_a	0	-
					S_6S_x	0	-

Cigany 59 ($S_{6c}S_9S_aS_b$) x Gold (S_3S_6)

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square [d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	16:20	0.25	0.62	S_3	16	$S_3S_9S_a$ (2); $S_3S_6S_b$ (4); $S_3S_9S_b$ (3);
S_6 / S_{6c}	1:1	29:7	12.25	0.0005			$S_3S_6S_9$ (2); $S_3S_9S_x$ (1); $S_3S_6S_a$ (3);
	3:1	29:7	0.33	0.57			$S_3S_aS_b$ (1)
S_9	1:1	22:14	1.36	0.24	S_6	20	$S_6S_aS_x$ (5); $S_6S_9S_b$ (4); $S_6S_9S_a$ (6);
S_a	1:1	18:18	0.03	0.86			$S_6S_9S_x$ (4); $S_6S_aS_b$ (1)
S_b	1:1	13:23	2.25	0.13			

Gold (S_3S_6) x Cigany 59 ($S_{6c}S_9S_aS_b$)

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square [d]	Probability	Possible male gametes	No. Successful	Genotypes exhibited (no.)
S_3	1:1	17:23	0.63	0.4274	S_9S_a	17	$S_3S_9S_a$ (9); $S_6S_9S_a$ (8)
S_6 / S_{6c}	1:1	23:17	0.63	0.4274	S_9S_b	15	$S_6S_9S_b$ (9); $S_3S_9S_b$ (6)

(Continued)

	3:1	23:17	5.63	0.0177	S_aS_b [e]	8	$S_6S_aS_b$ (6); $S_3S_aS_b$ (2)
S_9	1:1	32:8	13.2	0.0003	S_6S_9 [e]	0	-
S_a	1:1	25:15	2.03	0.1542	S_6S_a	0	-
S_b	1:1	23:17	0.63	0.4274	S_6S_b	0	-

Cigany 59 ($S_{6c}S_9S_aS_b$) x Chelan (S_3S_9)

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square [d]	Probability	Possible male gametes	No. Successful	Genotypes exhibited (no.)
S_3	1:1	7:0	5.14	0.02	S_3	7	$S_3S_9S_a$ (3); $S_3S_9S_b$ (2);
	1:0	7:0	-	-			$S_3S_aS_b$ (1); $S_3S_6S_a$ (1);
S_9	1:1	5:2	1.42	0.23	S_9	0	-
	3:1	5:2	0.05	0.82			
S_{6c}	1:1	1:6	2.28	0.13			
S_a	1:1	5:2	1.42	0.23			
S_b	1:1	3:4	0.00	1.00			

Rheinische Schattenmorelle (S_6 $S_{13'}S_aS_b$) x Gold (S_3S_6)

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square [d]	Probability	Possible male gametes	No. Successful	Genotypes exhibited (no.)
S_3	1:1	31:0	29.0	<0.0001	S_3	31	$S_3S_6S_a$ (10); $S_3S_6S_b$ (8); $S_3S_bS_{13'}$ (6);
	1:0	31:0	-	-			$S_3S_aS_{13'}$ (4); $S_3S_6S_{13'}$ (2); $S_3S_aS_b$ (1)
S_6	1:1	20:11	2.06	0.15	S_6	0	-
	3:1	20:11	1.30	0.25			
$S_{13'}$	1:1	12:19	1.16	0.28			
S_a	1:1	15:16	0.00	1.00			
S_b	3:1	15:16	0.00	1.00			

(Continued)

Gold (S_3S_6) x Rheinische Schattenmorelle ($S_6 S_{13}S_aS_b$)

	S-haplotype segregation				Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes exhibited (no.)
S_3	1:1	6:7	0.00	1.00	$S_bS_{13'}$	7	$S_3S_bS_{13'}$ (5); $S_6S_bS_{13'}$ (2)
S_6	1:1	7:6	0.00	1.00	$S_aS_{13'}$	6	$S_6S_aS_{13'}$ (5); $S_3S_aS_{13'}$ (1)
	3:1	7:6	2.08	0.15	S_aS_b[e]	0	-
$S_{13'}$	1:1	13:0	11.1	0.0009	S_6S_a	0	-
S_a	1:1	6:7	0.00	1.00	S_6S_b	0	-
S_b	1:1	7:6	0.00	1.00	$S_6S_{13'}$[e]	0	-

Montmorency ($S_6 S_{13}S_aS_x$[b]) x Gold (S_3S_6)

	S-haplotype segregation				Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square[d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	55:0	53.0	<0.0001	S_3	55	$S_3S_6S_x$ (15); $S_3S_6S_a$ (11); $S_3S_{13'}S_x$ (11); $S_3S_aS_{13'}$ (8); $S_3S_aS_x$ (7); $S_3S_6S_{13'}$ (3)
	1:0	55:0	-	-			
S_6	1:1	29:26	0.07	0.79	S_6	0	-
	3:1	29:26	13.39	0.0003			
$S_{13'}$	1:1	22:33	1.82	0.18			
S_a	1:1	26:29	0.07	0.79			

(Continued)

Gold (S_3S_6) x Montmorency ($S_6S_{13'}S_aS_x$[b])

S-haplotype segregation					Types and no. of successful male gametes		
S-haplotype	Expected ratio (P:A)[c]	Observed ratio (P:A)	Chi square [d]	Probability	Possible male gametes	No. Successful	Genotypes / phenotypes exhibited (no.)
S_3	1:1	8:7	0.00	1.00	$S_{13'}S_x$	9	$S_3S_{13'}S_x$ (5); $S_6S_{13'}S_x$ (4)
S_6	1:1	7:8	0.00	1.00	$S_aS_{13'}$	5	$S_3S_aS_{13'}$ (3); $S_6S_aS_{13'}$ (2)
	3:1		4.49	0.03	S_aS_x[e]	1	$S_6S_aS_x$ (1)
$S_{13'}$	1:1	14:1	9.6	0.002	S_6S_a	0	-
S_a	1:1	6:9	0.23	0.63	$S_6S_{13'}$[e]	0	-
					S_6S_x	0	-

[a] The *S*-genotype is presented when possible. If only two different *S*-haplotypes were detected in the progeny, the *S*-phenotype is presented with S_x representing either the double dosage of an *S*-haplotype or the presence of a null allele.

[b] For those sour cherry trees which have fewer than four different *S*-haplotypes, S_x is used to designate the fourth S-haplotype. S_x may either represent a *S*-haplotype in double dose or a null allele.

[c] Each *S*-haplotype was first tested for fit to a segregation ratio expected for a functional *S*-haplotype present in a single copy (1 present: 1 absent). If rejected, the segregation was tested for fit to a ratio expected for a non-functional *S*-haplotype (3:1 or 1:0).

[d] The Yates correction factor was used to account for the small population sizes in some populations.

[e] Sour cherry is an allotetraploid that undergoes occasional multivalent formation during meiosis. These gametes represent the rare combination of two *S*-haplotypes from homologous chromosomes, which require multivalent formation.

(Continued)

Emperor Francis (EF) x Surefire: Both the S_3- and S_4-haplotypes segregated 1 present: 1 absent in the 37 progeny, and no progeny inherited both the S_3 and S_4-haplotypes. These findings indicate that Surefire pollen containing the functional S_4 pollen-*S* product was rejected in a *S*-haplotype-specific manner by the S_4-RNase in the EF styles. The S_{13}-haplotype was present in nearly all progeny because it normally pairs with the S_4-haplotype during meiosis I. Since S_4-containing pollen is specifically rejected, all successful pollen will contain its homolog, $S_{13'}$, unless tetrasomic inheritance occurred to allow the creation of S_aS_x pollen.

Újfehértói fürtös (UF) x Schmidt: All 45 progeny contained the S_2-haplotype, and therefore all progeny resulted from fertilization of UF by Schmidt pollen that contained the S_2-haplotype. This indicates that pollen containing the S_4 pollen-*S* product were recognized and destroyed in a *S*-haplotype-specific manner by a functional S_4-RNase in UF styles.

Emperor Francis (EF) x Újfehértói fürtös (UF): Both the S_3- and S_4-haplotypes segregated 1 present: 1 absent in the 36 progeny, and no progeny inherited both S_3 and S_4-haplotypes. These findings indicate that UF pollen containing the functional S_4 pollen-*S* product was rejected in a *S*-haplotype-specific manner by the S_4-RNase in the EF styles. The S_e-haplotype was present in nearly all progeny because it normally pairs with the S_4-haplotype during meiosis I. Since S_4-containing pollen is specifically rejected, all successful pollen will contain its homolog, S_e, unless tetrasomic inheritance occurred to allow the creation of S_dS_x pollen.

Érdi Bőtermő (EB) x Emperor Francis (EF): All ten progeny contained the S_3-haplotype, thus all progeny resulted from fertilization of EB by EF pollen that contained the S_3-haplotype. This indicates that pollen containing the S_4 pollen-*S* product were recognized and destroyed in a *S*-haplotype-specific manner by a functional S_4-RNase in EB styles. The cDNA sequence of the *S_4-RNase* from EB was previously shown to be identical to the *S_4-RNase* of sweet cherry (Hauck et al., 2002), thus the finding that they are functionally identical is not surprising.

Emperor Francis (EF) x Érdi Bőtermő (EB): Both the S_3- and S_4-haplotypes segregated 1 present: 1 absent in the 20 progeny, and no progeny inherited both the S_3 and S_4-haplotypes. These findings indicate that EB pollen containing the functional S_4 pollen-*S* product was rejected in a *S*-haplotype-specific manner by the S_4-RNase in the EF styles. The S_{6m}-haplotype was present in nearly all progeny because it normally pairs with the S_4-haplotype during meiosis I. Since S_4-containing pollen is specifically rejected, all successful pollen will contain its homolog, S_{6m}, unless tetrasomic inheritance occurred to allow the creation of S_aS_x pollen.

Érdi Bőtermő (EB) x Gold: The S_3-haplotype segregated 1 present: 1 absent in the 33 progeny. The S_6-haplotype segregated 3 present: 1 absent. These findings indicate that all Gold pollen, regardless of whether they contain S_3 or S_6, are capable of fertilizing EB. Gold contains a functional *pollen-S_6* gene; therefore, the inability of EB to reject S_6 pollen indicates that the S_{6m}-RNase of EB is not functional. This is consistent with the

previous characterization of the S_{6m}-haplotype (Yamane et al., 2003). The S_{6m}-haplotype contains an insertion of approximately 2600 bp in the promoter region of the *S-RNase*, which prevented its expression.

Gold x Érdi Bőtermő (EB): Both the S_3- and S_6-haplotypes segregated 1 present: 1 absent in the 14 progeny, and no progeny inherited both the S_3 and S_6-haplotypes. These findings indicate that EB pollen containing the functional S_{6m} pollen-*S* product was rejected in a *S*-haplotype-specific manner by the S_6-RNase in the Gold styles. This is consistent with the previous characterization of the S_{6m}-haplotype (Yamane et al., 2003). Whereas the *S_{6m}-RNase* was not expressed, the expression pattern and DNA sequence of the *S_{6m}-SFB* was identical to the *S_6-SFB*. The S_4-haplotype was present in nearly all progeny because it normally pairs with the S_{6m}-haplotype during meiosis I. Since S_{6m}-containing pollen is specifically rejected, all successful pollen will contain its homolog, S_4, unless tetrasomic inheritance occurred to allow the creation of S_aS_x pollen.

Cigány 59 x Gold: The S_3-haplotype segregated 1 present: 1 absent in the 36 progeny, and the S_6-haplotype segregated 3 present: 1 absent. Both of these findings indicate that all Gold pollen, regardless of whether they contain S_3 or S_6, are capable of fertilizing Cigány 59. Gold contains a functional pollen-S_6 gene; therefore, the inability of Cigány 59 to reject S_6 pollen indicates that the S_{6c}-RNase of Cigány 59, similar to the S_{6m}-RNase of EB, is not functional.

Gold x Cigány 59: Both the S_3- and S_6-haplotypes segregated 1 present: 1 absent in the 40 progeny, and no progeny inherited both the S_3 and S_6-haplotypes. These findings indicate that Cigány 59 pollen containing the functional S_{6c} pollen-*S* product was rejected in a *S*-haplotype-specific manner by the S_6-RNase in the Gold styles. The S_9-haplotype was present in nearly all progeny because it normally pairs with the S_{6c}-haplotype during meiosis I. Since S_{6c}-containing pollen is specifically rejected, all successful pollen will contain its homolog, S_9, unless tetrasomic inheritance occurred to allow the creation of S_aS_b pollen.

Cigány 59 x Chelan: All seven progeny contained the S_3-haplotype, and therefore all progeny resulted from fertilization of Cigány 59 by Chelan pollen that contained the S_3 pollen-*S* product. Thus, pollen containing the S_9 pollen-S product were recognized and destroyed in a *S*-haplotype-specific manner by a functional S_9-RNase in Cigány 59 styles.

Rheinische Schattenmorelle (RS) x Gold: All 31 progeny contained the S_3-haplotype, and therefore all progeny resulted from fertilization of RS by Gold pollen that contained the S_3-haplotype. Pollen containing the S_6 pollen-S product were recognized and destroyed in a *S*-haplotype-specific manner by a functional S_6-RNase in RS styles. The cDNA sequence of the *S_6-RNase* from RS was previously shown to be identical to the *S_6-RNase* of sweet cherry (Hauck et al., 2002), thus the finding that they are functionally identical is not surprising.

Gold x Rheinische Schattenmorelle (RS): Both the S_3- and S_6-haplotypes segregated 1 present: 1 absent in the 13 progeny, and no progeny inherited both S_3 and S_6-haplotypes. These findings indicate that RS pollen containing the functional S_6 pollen-*S* product was rejected in a *S*-haplotype-specific manner by the S_6-RNase in the Gold styles. The $S_{13'}$-haplotype was present in all progeny because it normally pairs with the S_6-haplotype during meiosis I. Since S_6-containing pollen is specifically rejected, all successful pollen will contain its homolog, $S_{13'}$ unless tetrasomic inheritance occurred to allow the creation of S_aS_b pollen.

Montmorency x Gold: All 55 progeny contained the S_3-haplotype, and therefore all progeny resulted from fertilization of Montmorency by Gold pollen that contained the S_3-haplotype. Thus, pollen containing the S_6 pollen-S product were recognized and destroyed in a *S*-haplotype-specific manner by a functional S_6-RNase in Montmorency styles.

Gold x Montmorency: Both the S_3- and S_6-haplotypes segregated 1 present: 1 absent in the 15 progeny, and no progeny inherited both S_3 and S_6-haplotypes. These findings indicate that Montmorency pollen containing the functional S_6 pollen-*S* product was specifically rejected in a *S*-haplotype-specific manner by the S_6-RNase in the Gold styles. The $S_{13'}$-haplotype was present in nearly all progeny because it normally pairs with the S_6-haplotype during meiosis I. Since S_6-containing pollen is specifically rejected, all successful pollen will contain its homolog, $S_{13'}$, unless tetrasomic inheritance occurred to allow the creation of S_aS_x pollen.

Sour cherry self-populations

The segregation of *S*-haplotypes in sour cherry self-populations was analyzed to determine the functionality of *S*-haplotypes for which no sweet cherry tester was available ($S_{13'}$, S_a, S_b, S_d and S_e). The segregation of every *S*-haplotype in each of the self-populations is summarized in Table 4.6. The segregation data that is most critical for dissecting the genetic control of SI in sour cherry is described below.

Rheinische Schattenmorelle (RS) self-population: Following self-pollination of RS, 54 progeny were genotyped to determine the segregation of the four *S*-haplotypes in RS. The segregation of the S_6- and S_b-haplotypes fit 1 present: 1 absent ratios, indicating the presence of functional *S*-haplotypes in single copies. Pollen containing either of these functional pollen-*S* products was likely rejected in a *S*-haplotype-specific manner by the cognate functional *S*-RNases in RS styles. Both the S_a-and S_{13}-haplotypes were present in each of the 54 progeny, indicating that only pollen containing both S_a and $S_{13'}$ were capable of self-fertilization. The S_a and $S_{13'}$ pollen-*S* products were not recognized and degraded in a *S*-haplotype-specific manner by the S_a and $S_{13'}$ RNases, respectively. A schematic representation of this self-pollination can be found in the Appendix (Figure B.3).

Érdi Bőtermő (EB) self-population: Following self-pollination of EB, eight progeny were genotyped to determine the segregation of the three detectable *S*-haplotypes in EB. The S_4-haplotype segregated 1 present: 1 absent, indicating it is functional and present in a

Table 4.6: Sour cherry self-pollinations to test the functionality of $S_{13'}$, S_a, S_b, S_c, S_d and S_e.

Rheinische Schattenmorelle (S_6 $S_{13'}$,S_aS_b) self-pollinated

S-haplotype segregation					Progeny phenotypes obtained	
S-haplotype	Expected ratio [a]	Observed ratio	Chi square	Probability	Progeny Phenotype	No. observed
S_6	1:1	28:26	0.02	0.89	S_6 $S_{13'}$,S_aS_x	20
$S_{13'}$	1:1	54:0	52.0	<0.0001	$S_{13'}$,$S_aS_bS_x$	15
	1:0	54:0	-	-	$S_{13'}$,$S_aS_xS_x$	11
S_a	1:1	54:0	52.0	<0.0001	$S_6S_{13'}$,S_aS_b	8
	1:0	54:0	-	-		
S_b	1:1	23:31	0.91	0.34		

Erdi Botermo ($S_4S_{6m}S_aS_x$[b]) self-pollinated

S-haplotype segregation					Progeny phenotypes obtained	
S-haplotype	Expected ratio [a]	Observed ratio	Chi square	Probability	Progeny Phenotype	No. observed
S_4	1:1	4:4	0.13	0.72	$S_4S_{6m}S_aS_x$	4
S_{6m}	1:0	8:0	-	-	$S_{6m}S_aS_xS_x$	4
S_a	1:1	8:0	6.13	0.01		
	1:0	8:0	-	-		

Cigany 59 ($S_{6c}S_9S_aS_b$) self-pollinated

S-haplotype segregation					Progeny phenotypes obtained	
S-haplotype	Expected ratio [a]	Observed ratio	Chi square	Probability	Progeny Phenotype	No. observed
S_{6c}	1:0	37:0	-	-	$S_{6c}S_aS_bS_x$	14
S_9	1:1	14:23	1.73	0.19	$S_{6c}S_9S_aS_b$	9
S_a	1:1	37:0	35.0	<0.0001	$S_{6c}S_aS_xS_x$	9
	1:0	37:0	-	-	$S_{6c}S_9S_aS_x$	5
S_b	1:1	23:14	1.73	0.19		

Surefire (S_4 $S_{13'}$,S_aS_x[b]) self-pollinated

S-haplotype segregation					Progeny phenotypes obtained	
S-haplotype	Expected ratio [a]	Observed ratio	Chi square	Probability	Progeny Phenotype	No. observed
S_4	1:1	21:15	0.69	0.41	S_4 $S_{13'}$,S_aS_x	21
S_a	1:1	36:0	34.0	<0.0001	$S_{13'}$,$S_aS_xS_x$	15
	1:0	36:0	-	-		
$S_{13'}$	1:1	36:0	34.0	<0.0001		
	1:0	36:0	-	-		

(Continued)

Montmorency ($S_6 S_{13}$,S_aS_x[b]) self-pollinated

S-haplotype segregation					Progeny phenotypes obtained	
S-haplotype	Expected ratio [a]	Observed ratio	Chi square	Probability	Progeny Phenotype	No. observed
S_6	1:1	72:63	0.47	0.49	S_6S_{13},S_aS_x	67
S_a	1:1	131:4	117.6	<0.0001	S_{13},$S_aS_xS_x$	60
	1:0	131:4	-	-	S_6S_{13},S_xS_x	3
S_{13},	1:1	131:4	117.6	<0.0001	$S_6S_aS_xS_x$	2
	1:0	131:4	-	-	$S_aS_xS_xS_x$	2
					S_{13},$S_xS_xS_x$	1

Újfehértói fürtös ($S_4S_dS_eS_x$[b]) self-pollinated

S-haplotype segregation					Progeny phenotypes obtained	
S-haplotype	Expected ratio [a]	Observed ratio	Chi square	Probability	Progeny Phenotype	No. observed
S_4	1:1	60:42	2.83	0.09	$S_4S_dS_eS_x$	57
S_d	1:1	98:4	84.8	<0.0001	$S_dS_eS_xS_x$	41
	1:0	98:4	-	-	$S_4S_eS_xS_x$	3
S_e	1:1	102:0	100.0	<0.0001	$S_eS_xS_xS_x$	1
	1:0	102:0	-	-		

[a] Each *S*-haplotype was first tested for fit to a segregation ratio expected for a functional *S*-haplotype present in a single copy (1 present: 1 absent). If rejected, the segregation was tested for fit to a ratio expected for a non-functional *S*-haplotype (1:0).

[b] For those sour cherry trees which have fewer than four different *S*-haplotypes, S_x is used to designate the fourth *S*-haplotype. S_x may either represent a *S*-haplotype in double dose or a null allele.

single copy. Pollen containing the functional pollen-S_4 product was likely rejected in a *S*-haplotype-specific manner by the functional S_4-RNase in EB styles. Both the S_{6m}- and S_a-haplotypes were present in each of the eight progeny, indicating that only pollen containing both S_{6m} and S_a were capable of self-fertilization. The S_{6m} and S_a pollen-*S* products were not recognized and degraded in a *S*-haplotype-specific manner by the S_{6m} and S_a RNases, respectively. A schematic representation of this self-pollination can be found in the Appendix (Figure B.4).

Cigány 59 self-population: Following self-pollination of Cigány 59, 37 progeny were genotyped to determine the segregation of the four detectable *S*-haplotypes in Cigány 59. The segregation of the S_9- and S_b-haplotypes fit 1 present: 1 absent ratios, indicating the presence of functional *S*-haplotypes in single copies. Pollen containing either of these functional pollen-S products was likely rejected in a *S*-haplotype-specific manner by the cognate functional S-RNases in Cigány 59 styles. Both the S_{6c}-and S_a-haplotypes were present in each of the 37 progeny, indicating that only pollen containing both S_{6c} and S_a are capable of self-fertilization. The S_{6c} and S_a pollen-*S* products were not recognized and degraded in a *S*-haplotype-specific manner by the S_{6c} and S_a RNases, respectively. A schematic representation of this self-pollination can be found in the Appendix (Figure B.5).

Surefire self-population: Following self-pollination of Surefire, 36 progeny were genotyped to determine the segregation of the three detectable *S*-haplotypes in Surefire. The S_4-haplotype segregated 1 present: 1 absent, indicating it is functional and present in

a single copy. Pollen containing the functional pollen S_4- product was likely rejected in a *S*-haplotype-specific manner by the functional S_4-RNases in Surefire styles. Both the S_a- and $S_{13'}$-haplotypes were present in each of the 36 progeny, indicating that only pollen containing both S_a and $S_{13'}$ are capable of self-fertilization. The S_a and $S_{13'}$ pollen-*S* products were not recognized and degraded in a *S*-haplotype-specific manner by the S_a and $S_{13'}$ RNases, respectively. A schematic representation of this self-pollination can be found in the Appendix (Figure B.6).

Montmorency self-population: Following self-pollination of Montmorency, 135 progeny were genotyped to determine the segregation of the three detectable *S*-haplotypes in Montmorency. The S_6-haplotype segregated 1 present: 1 absent, indicating it is functional and present in a single copy. Pollen containing the functional pollen S_6-product was likely rejected in a *S*-haplotype-specific manner by the functional S_6-RNases in Montmorency styles. Both the S_a- and $S_{13'}$-haplotypes segregated 131 present: 4 absent. The majority of progeny inherited both S_a and $S_{13'}$, indicating that the S_a and $S_{13'}$ pollen-*S* products could not be recognized and degraded in an *S*-haplotype-specific manner by the S_a and $S_{13'}$ RNases, respectively. However, four progeny were the result of fertilization by pollen containing S_a and S_x, and another four progeny were the result of fertilization by pollen containing $S_{13'}$ and S_x, suggesting that the S_x-haplotype is also non-functional, but possibly linked to a gene that is deleterious for pollen growth, thus making pollen tubes containing S_x less competitive. A schematic representation of this self-pollination can be found in the Appendix (Figure B.7).

Újfehértói fürtös (UF) self-population: Following self-pollination of UF, 102 progeny were genotyped to determine the segregation of the three detectable *S*-haplotypes in UF. The S_4-haplotype segregated 1 present: 1 absent, indicating it is functional and present in a single copy. Pollen containing the functional pollen S_4- product was likely rejected in a *S*-haplotype-specific manner by the functional S_4-RNases in UF styles. The S_e-haplotype was present in each of the 102 progeny whereas S_d segregated 98 present: 4 absent. The majority of progeny inherited both S_d and S_e, indicating that the S_d and S_e pollen-*S* products could not be recognized and degraded in an *S*-haplotype-specific manner by the S_d and S_e RNases, respectively. However, four progeny were the result of fertilization by pollen containing S_e and S_x, suggesting that the S_x-haplotype is also non-functional, but possibly linked to a gene that is deleterious for pollen growth, thus making pollen tubes containing S_x less competitive. A schematic representation of this self-pollination can be found in the Appendix (Figure B.8).

Hypothesis verification

These genetic analyses led to the formation of a hypothesis for the genetic control of SI and SC in sour cherry, stating that a match between a functional pollen-*S* product in the pollen and a functional *S*-RNase in the style will result in rejection of the pollen. Pollen rejection will occur whether there are one or two functional matches. However, if there are no functional matches, the pollen will not be rejected. To test this hypothesis, the SI or SC phenotypes of 92 sour cherry selections was determined via observation of pollen tube growth down self-pollinated styles and compared with predictions based on

their *S*-genotypes. The results are summarized in Table 4.7. Ninety-one of the 92 predictions were accurate. The one inaccurate prediction has not been replicated.

Discussion

The segregation of *S*-haplotypes in various inter-specific crosses and self-populations was analyzed to determine the functionality of the *S*-haplotypes in six SC sour cherry selections in order to gain information on the cause of the partial breakdown of SI in this tetraploid species. The data suggest that functional and non-functional *S*-haplotypes are present in each of the examined SC trees. A hypothesis explaining the genetic control of SI and SC in sour cherry was developed and verified through crossing experiments. The implications of these findings are discussed below.

Three of the *S*-haplotypes, S_4, S_6 and S_9, were fully functional and identical to *S*-haplotypes found in sweet cherry, as initially hypothesized based on RFLP banding patterns (Yamane et al., 2001). However, the definitive proof that they are identical is that they can carry out *S*-haplotype-specific rejection in crosses with trees containing the same *S*-haplotype. Observation of pollen tube growth in reciprocal inter-specific crosses between Crisana ($S_1S_4S_d$) and Rainier (S_1S_4) suggested that the S_1 and S_4-haplotypes were fully functional and identical to the sweet cherry counterparts (Hauck et al., 2002). The observation of *S*-haplotype segregation in inter-specific crosses and self-pollinations presented in the current study confirmed that these three *S*-haplotypes were functional and identical to the sweet cherry counterparts.

Table 4.7: The *S*-genotype, SI or SC predictions based on the *S*-genotype, and the SI or SC phenotype of 92 sour cherry selections used to test the validity of the hypothesis for the genetic control of SI and SC in sour cherry.

Progeny S-genotype	Parents [a]	No. of Individuals	SI/SC prediction	SI/SC phenotype
$S_{13}S_aS_dS_e$	UF x Sure	8	SC	SC
$S_{13}S_aS_eS_x$	UF x Sure	4	SC	SC
$S_{13}S_aS_dS_x$	UF x Sure	2	SC	SC
$S_4S_{13}S_aS_x$	UF x Sure	10	SC	SC
$S_4S_{13}S_aS_e$	UF x Sure	3	SC	SC
$S_4S_{13}S_aS_d$	UF x Sure	2	SC	SC
$S_{13}S_aS_eS_x$	UF x RS	1	SC	SC
$S_{13}S_dS_eS_x$	UF x RS	4	SC	SC
$S_6S_{13}S_eS_x$	UF x RS	2	SC	SC
$S_{13}S_bS_dS_e$	UF x RS	1	SC	SC
$S_4S_6S_bS_x$	UF x RS	1	SI	SI
$S_4S_6S_bS_d$	UF x RS	3	SI	SI
$S_aS_dS_eS_x$	UF x Mont	1	SC	SC
$S_{13}S_aS_dS_x$	UF x Mont	1	SC	SC
$S_aS_dS_eS_x$	UF x Mont	3	SC	2 SC, 1 SI
$S_4S_6S_aS_e$	UF x Mont	1	SC	SC
$S_4S_6S_{13}S_d$	UF x Mont	1	SC	SC
$S_4S_6S_aS_x$	UF x Mont	2	SI	SI
$S_4S_{13}S_aS_b$	RS x EB	4	SC	SC
$S_4S_{13}S_aS_x$	RS x EB	17	SC	SC
$S_4S_6S_{13}S_a$	RS x EB	8	SC	SC
$S_4S_6S_aS_b$	RS x EB	5	SI	SI
$S_4S_6S_bS_x$	RS x EB	8	SI	SI

[a] UF = Újfehértói fürtös; Sure = Surefire; RS = Rheinische Schattenmorelle; Mont = Montmorency; EB = Érdi Bőtermő

The segregation of the S_b-haplotype in the RS and Cigány 59 self-populations indicate that it is also a fully functional allele that can carry out *S*-haplotype specific rejection. Although, to date, we have not found an identical *S*-haplotype in sweet cherry, it is possible that the S_b-haplotype does exist in wild sweet cherry germplasm. Alternately, it is possible that this *S*-haplotype was inherited from the other parent of the allotetraploid sour cherry, *P. fruticosa*. There has not been an in depth investigation of SI in *P. fruticosa*, making it currently impossible to know whether the S_b-haplotype is originally from sweet cherry or *P. fruticosa*. A third possibility is that this *S*-haplotype was formed more recently in sour cherry, and does not exist in either of the progenitor species.

Each of the six SC selections included in this study contain at least one functional *S*-haplotype that is capable of carrying out *S*-haplotype-specific rejection of pollen. RS contains both S_6 and S_b, Cigány 59 contains S_9 and S_b, EB contains S_4, Surefire contains S_4, UF contains S_4 and Montmorency contains S_6. This implies that each of these selections contains all the necessary machinery for initiating and carrying out an SI reaction. Thus, the breakdown of SI in these plants was not due to a mutation of one of these components, but instead must have been caused by a mutation in one of the *S*-haplotype specificity components, either the *S-RNase* or the *pollen-S*.

In addition, RS (S_6 and S_b), Cigány 59 (S_9 and S_b), and EB (S_4 and S_{6m}) contain two *S*-haplotypes with functional *pollen-S* genes. Thus, it would be expected that each of these three SC selections would produce some pollen that contains two functional *pollen-S* genes. If the breakdown of SI in sour cherry were caused by the competition of pollen-*S* products in heteroallelic pollen, then these pollen types would be able to successfully

fertilize any styles, including those containing the counterpart *S*-RNases. However, in each case, these *S*-haplotypes were never inherited by progeny via pollen in these crosses. For instance, when RS was used to pollinate Gold styles, none of the progeny inherited the S_6 haplotype from RS. This result indicates that pollen containing S_6 was specifically rejected in Gold styles, regardless of whether there was only one functional pollen-*S* product (either S_6S_a or $S_6S_{13'}$) or two functional pollen-*S* products (S_6S_b). Two hypotheses that could defend the competitive interaction theory are that either gametes containing S_6 and S_b never form due to the pairing of chromosomes during meiosis or these gametes are not viable due to the linkage of one of these *S*-haplotypes to a deleterious gene. However, in the reciprocal cross in which Gold was used to pollinate RS, 8 out of 31 progeny inherited the S_6 and S_b from RS. Approximately one quarter of the progeny inherited this *S*-haplotype combination from RS, which would be expected assuming strict disomic inheritance of alleles that are on homoeologous chromosomes. Thus, gametes containing this combination of *S*-haplotypes occur at a high frequency. The fact that no RS pollen containing the S_6-haplotype was inherited in the Gold x RS cross suggests that heteroallelic pollen retains its ability to trigger an SI reaction and that SI does not breakdown in sour cherry due to a competition between pollen-*S* products in heteroallelic pollen.

Since the breakdown of SI in sour cherry is not caused by competition between pollen-*S* products within a pollen tube or by mutations in non-haplotype-specific modifier genes, an alternate explanation for the observed breakdown is necessary. The data suggests that the breakdown of SI in sour cherry is due to the accumulation of non-functional *S*-haplotypes that have lost the function of at least one of the *S*-haplotype

specificity components, *S-RNase* or *pollen-S*. Several *S*-haplotypes, specifically S_{6c}, S_{6m}, $S_{13'}$, S_a, S_d, and S_e, all appear to be non-functional. None of these *S*-haplotypes is capable of initiating an SI reaction, even if a corresponding *S*-haplotype was also present in the stylar parent.

In the case of S_{6c} and S_{6m}, it was possible to use reciprocal crosses with Gold, which is known to contain a fully functional S_6-haplotype, to determine if the *S-RNase* and/or the *pollen-S* gene were non-functional. As summarized in Table 4.5, when Gold was the maternal parent and either Cigány 59 or EB was the paternal parent, none of the progeny inherited a gamete containing either S_{6c} or S_{6m} from Cigány 59 or EB, respectively. This result suggests that the S_6-RNase in the Gold styles was able to specifically reject pollen containing either the S_{6c}- or S_{6m}-haplotype. Thus the pollen-*S* product must be functional and capable of triggering the SI reaction in both of these variants of the S_6-haplotype. When Cigány 59 or EB was pollinated with Gold pollen, the S_3-haplotype segregated 1:1 whereas the S_{6c} and S_{6m}-haplotypes each segregated 3:1. This result suggests that pollen containing either S_3 or S_6 was able to grow through Cigány 59 or EB styles. Gold contains a functional *pollen-S_6* gene, so the ability of S_6 pollen to successfully pollinate Cigány 59 or EB must be due to the S_{6c}- and S_{6m}-RNase genes being non-functional. Yamane et al. (2003) characterized the S_{6m}-haplotype from EB and discovered that the *S_{6m}-RNase* is not expressed in EB styles, probably because of a 2600 bp insertion in the putative promoter region of the *S_{6m}-RNase* gene. The sequence and expression of the *S_{6m}-SFB* was identical to that of the *S_6-SFB* from sweet cherry. Similar sequence and expression analyses of the S_{6c}-haplotype from Cigány 59 are underway.

It should also be possible to determine if the *S-RNase* or *pollen-S* gene is non-functional in the S_{13}-haplotype, since a functional counterpart exists in sweet cherry; however, appropriate crosses were not made due to the lack of availability of a S_{13} sweet cherry tester. Tobutt et al. (2004) reported that the sour cherry selections Marasca Piemonte, Marasca Savena and Morello Dutch, which all contain S_6 and S_{13}, reject pollen from the sweet cherry Noble (S_6S_{13}). This suggests that the S_{13} from these sour cherry selections contain a functional *S_{13}-RNase* that is similar to the S_{13} from sweet cherry. The reciprocal cross, however, was not reported. These results make it likely that $S_{13'}$ contains a functional *S_{13}-RNase* but a non-functional *pollen-S_{13}* gene. Molecular analysis of the S_{13}-haplotype is underway.

Since functional versions of the S_a, S_d and S_e-haplotypes are not known in sweet cherry, self-pollinations were used to conclude that each of these *S*-haplotypes were non-functional. An example of how this data is interpreted is as follows. The *S*-genotype of RS is $S_6 S_{13'}S_aS_b$. In the self-progeny, both the S_6 and S_b haplotypes segregate 1:1, whereas every progeny inherited both the S_a and $S_{13'}$ haplotypes. RS styles are hypothesized to contain functional S_6- and S_b-RNases; however, the presence of functional S_a- or S_{13}-RNases has not been confirmed. Since sour cherry is a segmental allopolyploid capable of occasional multivalent formation (Wang et al., 1998), six pollen types are possible from RS: S_6S_a, S_6S_b, $S_6 S_{13'}$, S_aS_b, $S_a S_{13'}$, and $S_b S_{13'}$. Both the S_6 and S_b pollen-*S* products are functional; however, the presence of a functional pollen-*S* product for S_a and $S_{13'}$ is not known. It is hypothesized that either the *S-RNase* or *pollen-S* gene for each of these two non-functional *S*-haplotypes is mutated, preventing specific rejection of these *S*-haplotypes. Since the S_6- and S_b-haplotypes are functional, any

pollen containing one or both of these *S*-haplotypes will be recognized and destroyed by the S_6- or S_b-RNases in the styles, respectively. Any pollen that does not contain either S_6 or S_b will not be recognized. Thus, all self-progeny should, and did, inherit the S_a- and S_{13}-haplotypes from pollen. Similar analyses were done for the S_d- and S_e-haplotypes.

Whereas the self-pollinations allowed the conclusion that S_a, S_d and S_e are all non-functional *S*-haplotypes, it would be necessary to find a functional counterpart to these *S*-haplotypes, either in sweet or sour cherry, before conclusions can be drawn as to whether mutations in the *S-RNase* or *pollen-S* gene cause a loss-of-function. Since functional versions of the S_a, S_d and S_e-haplotypes are currently undiscovered in sweet cherry, inter-specific crosses could not be used to determine whether the *S-RNase* and/or the *pollen-S* gene was non-functional. However, the cDNA and N-terminal amino acid sequences were reported for the S_a-RNase (Yamane et al., 2001; Hauck et al., 2002), suggesting that this protein is made. Although the functionality of the S_a-RNase is unknown, the fact that it is expressed suggests that the loss-of-function of the S_a-haplotype may be due to mutations in the *pollen-S* gene. We are currently in the process of cloning and sequencing the *S-RNase* and *SFB* genes, as well as their promoters, for the S_a, S_d and S_e-haplotypes to look for mutations. In addition, expression analyses will be conducted for both genes in each of these *S*-haplotypes. These types of molecular characterizations, similar to those done for the S_{6m}-haplotype (Yamane et al., 2003), should tell us which of the genes has been mutated in each of these non-functional *S*-haplotypes.

In this study, two selections, Cigány 59 and EB, contain non-functional S_6-haplotypes composed of a functional *pollen-S* gene and a non-functional *S-RNase* gene. Existing evidence suggests that these mutations were caused by independent events.

First, RFLP analysis following digestion with *Hin*dIII and probing with an *S-RNase* probe revealed different banding patterns for S_{6c} and S_{6m} (Yamane et al, 2001). The fragment corresponding to S_{6c} was similar in size to that of the functional S_6-haplotype from sweet cherry (approximately 5.8 kb), whereas the S_{6m} fragment was approximately 9 kb. Thus, it is likely that the loss-of-function is not caused by a large insertion, but rather a smaller insertion, deletion or a base substitution. In addition, PCR primers designed to amplify the 2600 bp insertion in the S_{6m}-*RNase* promoter (Yamane et al., 2003) do not amplify a band in Cigány 59 (data not shown). The occurrence of two independent mutations in the S_6-*RNase* gene in sour cherry could imply the presence of a mutational "hot spot". It is interesting to note that the distance between the S_6-*RNase* and S_6-*SFB* is only approximately 300 bp (Yamane et al., 2003), compared to other *S*-haplotypes that may have intergenic spaces as great as 40 kb (K. Ikeda, unpublished).

In four of the six sour cherry studies examined, only three *S*-haplotypes could be distinguished. In addition, the segregation data suggested that each of these *S*-haplotypes was present in a single copy. Thus, the fourth *S*-haplotype in these selections is hypothesized to be a null allele (S_{null}), consisting of a large deletion that included the *S-RNase*. The deletion of the *S-RNase* is consistent with the presence of only three fragments on a Southern upon hybridization with an *S-RNase* probe. S_{null} is expected to be non-functional due, at least, to the absence of a functional *S-RNase*. However, it is unknown whether the deletion includes the *pollen-S* gene. The presence of a functional *pollen-S* gene would allow pollen containing the S_{null} to be rejected by styles containing a cognate functional *S*-RNase, if one exists.

The ability of heteroallelic sour cherry pollen to trigger an SI reaction suggests that the identity of the *pollen-S* gene and/or the mechanism of SI in the Solanaceae may differ from that in *Prunus*, despite the belief that the *S-RNase* in the Solanaceae and Rosaceae are orthologous (Igic and Kohn, 2001) and the recent discovery that an F-box gene may be the pollen determinant in both the Solanaceae and *Prunus* (Entani et al., 2003; Ushijima et al., 2003; Sijacic et al., 2004). However, initial comparison of the different F-box genes suggests that they are not orthologous. In all species studied to date, multiple F-box genes are linked to the *S-RNase*; thus, it is possible that *Prunus* and the Solanaceae recruited different F-box genes (*SFB* vs *PiSLF*) to act as the *pollen-S* gene. An additional difference between gametophytic SI in *Prunus* and the Solanaceae is that, despite the identification of several PPMs through mutant screens, no PPMs caused by either the loss of expression of the *pollen-S* gene or the expression of a mutant *pollen-S* gene have been observed in the Solanaceae (Pandey 1967; van Gastel and de Nettancourt, 1975; Golz et al., 1999; Golz et al., 2000). Instead, all identified Solanaceous PPMs were caused by the duplication of the *pollen-S* gene. In comparison, at least two PPMs in *Prunus*, S_4' from sweet cherry and S_f from *P. mume* (Ushijima et al., 2004) have been caused by mutation of the *pollen-S* gene. These findings suggest that the identity and function of the *pollen-S* gene may differ between the Solanaceae and *Prunus*. Furthermore, competitive interaction between pollen-*S* products is thought to cause a breakdown of SI in other Rosaceous genera, such as *Malus* and *Pyrus* (R. Tao, pers. comm.). Therefore, it is possible that not only does the identity and function of the *pollen-S* gene differ between the Solanaceae and *Prunus*, but also between *Prunus* and other Rosaceous genera.

The data presented in this research indicate that the cause of the partial breakdown of SI in sour cherry is due to the accumulation of non-functional *S*-haplotypes that contain mutations in one or both of the *S*-haplotype-specificity components, *S-RNase* or *pollen-S*, rather than competitive interaction between pollen *S*-products or due to mutations in a modifier gene. Therefore, our current hypothesis for the genetic control of SI and SC in sour cherry is that a match between a functional pollen-*S* product in the pollen and a functional *S*-RNase in the style will result in rejection of the pollen. Pollen rejection will occur whether there are one or two functional matches. However, if there are no functional matches, the pollen will not be rejected.

To test the validity of this hypothesis, the SI or SC phenotypes of 92 sour cherry selections was determined via observation of pollen tube growth in self-pollinated styles and compared with predictions based on their *S*-genotypes. Of these 92 predictions, 91 were accurate. The one incorrect prediction was for a selection that had the *S*-genotype $S_{13}S_dS_eS_x$. The presence of at least two non-functional *S*-haplotypes in this selection would make this tree SC, according to the hypothesis. The reason for the incorrect prediction is unclear and is currently being investigated. However, this result has not been replicated and the most likely explanation is that the genotyping and phenotyping were mistakenly conducted for different trees. This experiment will be replicated in the Spring of 2005.

Previously, the segregation of *S*-haplotypes and the SI or SC phenotype among progeny in a cross between RS and EB was reported (Hauck et al., 2002). At the time, we could not explain the observed segregation. However, with our current understanding of the functionality of the *S*-haplotypes in these two parents and our hypothesis

explaining the genetic control of SI and SC in sour cherry, we can now explain the observed segregation. All progeny that contained at least two non-functional *S*-haplotypes were SC, whereas all progeny that contained fewer than two non-functional *S*-haplotypes were SI. See Table B.3 and Figure B.1 for a complete list of progeny *S*-genotypes and SI or SC phenotypes.

The elucidation of the genetic control of SI and its breakdown in sour cherry has implications on our understanding of gametophytic SI and the effects of polyploidy on gametophytic SI, as discussed above, as well as on sour cherry breeding. Growers demand SC sour cherry cultivars due to inefficiencies related to growing SI cultivars, such as a reliance on bees for fruit set and the need to grow pollinator varieties in the orchard. Thus, one trait that breeders are concerned with is SC. With the knowledge of the genetic control of SI and SC in sour cherry, a breeder could use *S*-haplotype markers to predict whether a seedling is SI or SC, allowing the elimination of seedlings that are predicted to be SI. In addition, the breeder could predict whether a particular cross would result in SI progeny, SC progeny or a mixture of both types of progeny. Thus, breeders could save both time and orchard space.

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CHAPTER 5

CONCLUSIONS, GENERAL DISCUSSION AND FUTURE RESEARCH

Conclusions and General Discussion

The goal of the presented research was to determine the genetic control of self-incompatibility (SI) and self-compatibility (SC) in sour cherry. To accomplish this goal, it was first necessary to become familiar with the *S*-haplotypes that exist in sweet cherry, one of the progenitor species of sour cherry. RFLP analyses were used to determine the *S*-haplotype constitution of 22 sweet cherry selections. In total, the RFLP banding patterns were described for 14 different *S-RNase* alleles. Several new sweet cherry *S*-haplotypes have since been reported [S_{14}: Wiersma et al., (2001); S_{16}: Sonneveld et al., (2003); S_{23}, S_{24}, S_{25}: Wunsch and Hormaza (2004)]. As new *S*-haplotypes are reported, it will be possible to compare them with the sour cherry *S*-haplotypes to see if they are identical.

RFLP analyses were then conducted for a diverse set of 12 sour cherry selections to determine which *S*-haplotypes exist in sour cherry (Yamane et al., 2001). A total of 11 different *S*-haplotypes were identified using two different restriction enzymes. Of these, S_1, S_4, S_6, S_9 (called S_x) and S_{12} (called S_{13}) had identical banding patterns to *S*-haplotypes in sweet cherry, whereas S_{6m}, S_a, S_b, S_c, S_d and S_e had unique banding patterns. Whether the *S*-haplotypes that appeared to be in common were functionally similar remained unknown until reciprocal inter-specific crosses with sweet cherry were analyzed in this study (discussed later). Since the release of this paper, the S_c has been renamed $S_{13'}$ due to a report that several sour cherry trees contained the S_{13}-RNase protein (Tobutt et al., 2003) and the *S*-RNase amino acid sequences for S_c and S_{13} were identical. However, the $S_{13'}$ haplotype appears to be a non-functional version of S_{13}, probably due to a mutation in

the *pollen-S* gene. In addition, the S_e-haplotype is most likely a mutated version of S_1, containing a functional *S_1-RNase* and a non-functional *pollen-S_1* gene. This conclusion is based on the segregation of the S_1, S_3 and S_e, -haplotypes in reciprocal crosses with the sweet cherry Regina (S_1S_3) and the ability to amplify a fragment from S_e that is identical to S_1 using *S_1-RNase* allele specific primers and *S-RNase* consensus primers.

Observations of pollen tube growth in inter-specific crosses involving the sour cherry Crişana ($S_1S_4S_dS_x$) and the sweet cherry Rainier (S_1S_4) provided the first evidence that the S_1 and S_4-haplotypes in sour cherry, as identified by RFLP, were fully functional and identical to the S_1 and S_4 haplotypes in sweet cherry. The incompatibility of Crişana pollen with Rainier styles also provided the first evidence that the breakdown of SI in heteroallelic pollen observed in Solanaceous species does not occur in sour cherry, since Crişana should be able to produce some pollen containing two functional pollen-*S* products but is SI. If the competition between pollen-*S* products in heteroallelic pollen causes a breakdown of SI in *Prunus*, then Crişana should be SC.

Finally, the segregation of *S*-haplotypes in various inter-specific crosses and self-populations established that sour cherry is composed of a mixture of functional (S_1, S_4, S_6, S_9, and S_b) and non-functional (S_{6c}, S_{6m}, $S_{13'}$, S_a, S_d, and S_e) *S*-haplotypes. In addition, all sour cherry selections tested were capable of carrying out *S*-haplotype-specific rejection, indicating that the breakdown of SI in sour cherry was not caused by mutations in the machinery necessary to carryout an SI reaction, but rather in the specificity components, either the *S-RNase* or the *pollen-S* gene, themselves. The possibility that competitive interaction between pollen *S*-products causes the breakdown of SI in sour cherry was eliminated because of the overwhelming evidence against this hypothesis in the

segregation data. Instead, the genetic cause of SC in sour cherry is the accumulation of non-functional *S*-haplotypes that are incapable of triggering or initiating an SI reaction.

Taken together, the data support the "one-allele match" hypothesis. This hypothesis states that the genetic control of SI and SC in sour cherry is based solely on which *S*-haplotypes are present. Any time there is a match between a functional pollen-*S* product in the pollen and a functional *S*-RNase in the style, the pollen will be rejected. Pollen rejection will occur whether the pollen is homoallelic or heteroallelic and whether there are one or two functional matches. However, if there are no functional matches, the pollen will not be rejected.

These findings not only further our understanding of the genetic control of SI in sour cherry, they should aid sour cherry breeders and increase our knowledge of how polyploidy affects GSI. These impacts are discussed in more detail below.

Impact on sour cherry breeding

In order for growers to obtain fruit from an SI variety, pollinator trees must be inter-planted with the variety of interest and bees must be released to ensure proper cross-pollination. These problems associated with growing SI trees make SC cultivars highly desirable. Thus, breeders must select for SC trees in addition to improved fruit quality, tree quality and resistance traits. Currently, breeders must wait three to five years before flowers are available to test for SC. If the selection process could be done earlier, the breeder could eliminate all SI material, saving space in their orchard and evaluation time for seedlings that are SC. Until now, the genetic cause of SC was unknown, making it impossible to use molecular markers to predict the SI or SC phenotype of a seedling.

Now that the genetic cause of SC is known, breeders can look for trees that contain at least two of the identified non-functional *S*-haplotypes. Any seedling that contains fewer than two non-functional *S*-haplotypes will be SI. With this information, breeders can select for SC material immediately after germination of the seedling. In addition, a breeder can select parents that maximize the chances of obtaining SC progeny. For example, the pollination of a tree of *S*-genotype $S_4S_6S_9S_b$ (which is SI) with pollen from a tree that is $S_4S_6S_aS_c$ would result in 100% SC seedlings, since the only successful pollen would be S_aS_c, thus all progeny would also contain these two non-functional *S*-haplotypes. Alternately, a cross between two SC trees, $S_1S_4S_aS_d$ and $S_6S_9S_aS_e$, would result in a mixture of SI and SC progeny. Without understanding the genetic basis of SI and SC, the breeder might assume that the cross between two SC parents would result in more SC progeny than the cross between the SI and SC parent.

Chapter 4 presented the results from SI or SC phenotypic predictions for 92 trees from the MSU Clarksville Horticultural Research Station (CHES) based on their *S*-genotypes or *S*-phenotypes. In total, accurate predictions were made for 91 of the 92 trees. The one incorrect prediction was for a progeny from a cross between Újfehértói fürtös (UF) and Montmorency that had the *S*-genotype $S_cS_dS_eS_x$. This tree contains at least three non-functional *S*-haplotypes and should be SC, according to the "one-allele match" hypothesis. The cause of the inaccurate prediction is currently being investigated; however, it is very likely that this discrepancy was caused by lab error. For example, it is possible that the *S*-genotyping and SI/SC phenotyping were mistakenly conducted on different plants. However, a success rate of 91 out of 92 verifies the effectiveness of screening seedlings for SC types. In addition, the compatibility of 13

crosses between trees located at CHES (Table B.2) were predicted. In this case, 100% of the predictions were accurate.

One potential problem is if "new" germplasm is used for crosses. All the research presented in this dissertation is based on the investigation of the *S*-haplotypes that are present in 12 sour cherry selections. These 12 selections obviously do not contain every *S*-haplotype that exists in sour cherry. Thus, the use of other sour cherry germplasm could introduce new *S*-haplotypes. The functionality of these *S*-haplotypes is unknown, making the prediction of SI or SC difficult. Table B.1 includes nine progeny of trees that have never been studied [III 18(12) and Erdi Jubileum]. Six progeny were from a cross between UF and III 18(12), which was found to have a new *S*-haplotype, named S_f. Three addition progeny were from a cross between Montmorency and Erdi Jubileum, which also contained a new *S*-haplotype, named S_g. For two progeny from these crosses [27 9(12) and 27 8(58)], correct phenotype predictions could only be made if the S_f and S_g were assumed to be non-functional. The other seven progeny from these crosses could be predicted without any assumptions since they contained more than two non-functional *S*-haplotypes, regardless of whether or not the S_f and S_g are functional. In conclusion, breeders can accurately use markers to predict the SI or SC phenotype of seedlings as long as there is knowledge of the *S*-haplotypes that exist in the seedling. Novel *S*-haplotypes could be problematic for accurate SI or SC phenotype prediction of seedlings.

Implications for understanding the effect of polyploidy on GSI

Since the first observations of SC tetraploid plants in the 1930's (Crane and Lawrence, 1931), researchers have thought that GSI breaks down in response to an

increase in ploidy level. It was later hypothesized that the presence of two pollen-*S* products in a single pollen tube results in a competition and a loss of SI phenotype (Lewis, 1943). Recent experiments with *Petunia* verified that heteroallelic pollen loses its SI phenotype (Entani et al., 1999). In fact, Sijacic et al. (2004) took advantage of the competitive interaction phenomenon to prove that a recently cloned gene, *PiSLF*, was the long sought-after *pollen-S* gene in *Petunia inflata*.

Stebbins (1950) predicted that SC is more common in polyploids because SC is necessary for the establishment of an otherwise reproductively isolated plant (Stebbins, 1950; Miller and Veneble, 2000) and because polyploids can tolerate selfing due to less inbreeding depression (Lande and Schemske, 1985). Husband and Schemske (1997) provided evidence that the frequency of SC is higher in tetraploids than in diploids. However, a more recent comparison of ploidy level and the SI or SC phenotype of species among angiosperms led to the conclusion that the frequencies are not significantly different (Mable, 2004). These findings suggest that self-fertility is not a prerequisite for the establishment of a polyploid, as previously suggested by Stebbins (1950).

The results from the current study suggest that polyploidy itself does not directly cause SC in sour cherry, but instead can have an indirect effect on SI. The presence of six natural non-functional *S*-haplotypes in a set of 12 sour cherry selections is surprisingly high compared to the diploid sweet cherry, which carries no known natural non-functional *S*-haplotype. This suggests that the *S*-locus may be less stable in polyploids than in diploids. Whether the loss-of-function occurred immediately after polyploid formation due to an increase in transposition and other genome rearrangements

often seen in newly formed polyploids (Song et al., 1995; Shaked et al., 2001; Ozkan et al., 2001) or if it is by on-going loss-of-function mutations is unclear; however, the evidence suggests that the *S*-locus is less stable. Although the loss of GSI appears to be directly caused by the increase in ploidy in the Solanaceae, the loss of GSI may not always be directly caused by polyploidy, as evidenced by the current study of sour cherry.

In the Solanaceae, SI breaks down in response to the competitive interaction between pollen-*S* products in heteroallelic pollen. This phenomenon does not occur in *Prunus*, suggesting that the identity of the *pollen-S* gene and/or the GSI mechanism may differ between the Solanaceae and *Prunus*. This observed difference between the Solanaceae and *Prunus* was unexpected, given that phylogenetic analyses of the *S-RNase* gene suggest a common origin of the gene in the Solanaceae and *Prunus*, with the only major difference being the *S-RNase* gene from *Prunus* contains two introns while the *S-RNase* gene from all other Rosaceous species and all Solanaceous species contain only one (Igic and Kohn, 2001).

Similar phylogenetic analyses for the *pollen-S* gene have not been conducted. Initial comparison of the putative *pollen-S* gene from *Prunus* (*SFB*: Ushijima et al., 2003) and from *Antirrhinum* (*SFL*: Lai et al., 2002) suggests that they are not orthologous. Interestingly, no pollen-part mutant (PPM) caused by the mutation of the *pollen-S* gene has been identified in the Solanaceae (Pandey 1967; van Gastel and de Nettancourt, 1975; Golz et al., 1999; Golz et al., 2000). Instead, all identified PPMs in the Solanaceae were caused by the duplication of the *pollen-S* gene. In comparison, at least two PPMs in *Prunus*, S_4' from sweet cherry and S_f in *P. mume* (Ushijima et al., 2004) have been caused

by mutation of the *pollen-S* gene. These findings suggest that the identity and function of the *pollen-S* gene may differ between the Solanaceae and *Prunus*. Thus, it is possible that the Solanaceae and *Prunus* use a homologous style-determinant (*S-RNase*) but recruited different genes to act as the pollen-determinant (*SFB* vs *SLF*) in GSI.

Alternately, the breakdown of GSI due to competitive interaction between pollen-*S* products might be more likely in autotetraploids than in allotetraploids. The studies in the Solanaceae have mainly focused on artificially induced polyploids caused by the addition of colchicine, whereas sour cherry is a natural allotetraploid species. It is possible that the physical nature of autopolyploids and allopolyploids is the cause of the observed difference in heteroallelic pollen. For example, it is possible that *pollen-S* gene products from the different genomes of an allotetraploid do not interact and are not capable of competition, whereas the *pollen-S* gene products from within the same genome of an autotetraploids are capable of interacting and competing with one another.

Future Research

The presented research successfully determined the genetic control of SI and SC in sour cherry. However, several other questions have emerged during the past few years. Presented below is a list of questions that should be addressed and experiments that should be conducted to help answer those questions.

1. What is the molecular basis for the loss-of-function of each of the non-functional sour cherry *S*-haplotypes (S_{6c}, S_{13}, S_a, S_d and S_e)?

The following experiments will allow the detection of mutations in either the coding region or regulatory region of the genes from each non-functional S-haplotype. This is similar to the work done to characterize the loss-of-function mutation observed in the S_{6m}-haplotype from sour cherry (Yamane et al., 2003) and S_4-haplotype and S_f-haplotypes from sweet cherry and Japanese apricot, respectively (Ushijima et al., 2004).

1.a: Determine the cDNA sequence of each *S-RNase*

1.b: Isolate genomic clones containing *SFB* and determine the DNA sequence of *SFB*.

1.c: Use RT-PCR to determine whether each *S*-RNase or SFB is expressed in styles or pollen, respectively.

1.d: For those genes that are not expressed, sequence the promoter region to determine the cause of the observed loss of expression.

2. Are *P. fruticosa* individuals SI or SC and what *S*-haplotypes exist in *P. fruticosa*?

The existence of GSI in sweet cherry and sour cherry is well documented; however, the existence of GSI in *P. fruticosa*, the other progenitor of sour cherry, has not been documented. In addition, several sour cherry *S*-haplotypes have not been found in

sweet cherry and it is possible that they originate from *P. fruticosa*. A complete understanding of SI and its partial breakdown to SC in sour cherry depends on an understanding of SI in *P. fruticosa*. The following set of experiments will give initial insights on SI in *P. fruticosa*.

2A: Determine the SI or SC phenotype of a set of *P. fruticosa* selections from a diverse range of geographic locations.

2B: Use RFLP analyses to determine what *S*-haplotypes exist in this diverse set of *P. fruticosa* germplasm.

2C: Obtain cDNA sequences for *S-RNases* from *P. fruticosa* selections. Compare the *S-RNase* sequences with those from sour cherry.

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Appendices

APPENDIX A

DNA EXTRACTION PROTOCOL FOR CHERRY SEED

DNA extraction from cherry seed

1. Use hammer to break hard seed coat. Take off papery coating around cotyledons
2. Split apart the cotyledons (not necessary, but makes it easier to crush)
3. Grind in liquid nitrogen. Try to prevent tissue from "jumping out" of the mortar when crushing by covering the mortar with your hand
4. Quickly add 750ul of extraction buffer and transfer to 15ml orange cap tube. Add an additional 750ul buffer to the mortar and then transfer it to the same 15ml tube.

Extraction Buffer

Final concentration	Starting []	For 10 seed
200 mM Tris-HCl (pH 8.0)	1 M	3.6 ml
200 mM NaCl	5 M	720 ul
25 mM EDTA	.5 M	900 ul
.5% SDS	20%	450 ul
H2O		12.3 ml

5. Add 1.5ml of CTAB solution to each 15 ml tube

CTAB buffer solution

Final concentration	Starting []	For 10 seed
2% CTAB	5%	7.2 ml
100 mM Tris-HCl (pH 8.0)	1 M	1.8 ml
20 mM EDTA	.5 M	720 ul
1.4 M NaCl	5 M	5.04 ml
2% BME		360 ul
H2O		2.88 ml

6. Invert a couple times and incubate 10 minutes at room temp
7. Add 3 ml chloroform:isoamyl alcohol (24:1).
8. Centrifuge 6500 rpm in for 10 min and transfer supernatant to new tubes
9. If any white chunks are in this supernatant after transferring it to the new tube, re-centrifuge for an additional 10 minutes and transfer supernatant to new tube. Keep repeating until there are no more white chunks.
10. Add 2/3 volume of isopropanol (about 1.6 mls) and incubate at room temp for 10 min to precipitate DNA
11. Centrifuge at 6,500 rpm for 10 min. If there is no pellet, try re-centrifuging in the tabletop centrifuge for a couple additional minutes. Remove supernatant. Careful not to lose the little pellet
12. Wash DNA pellet with 70% ethanol, air dry, resuspend in about 40 ul TE, and add 1 ul RNase (10mg/ml)

APPENDIX B

COLLECTION OF SELF-INCOMPATIBILITY DATA AND SCHEMATIC REPRESENTATIONS OF OBSERVED *S*-HAPLOTYPE SEGREGATION DATA

Table B.1: The self-pollination of 60 sour cherry selections to test the validity of the hypothesis for the genetic control of SI and SC in sour cherry. Predictions were based on the knowledge of the *S*-genotypes of each tree.

Plant ID	Maternal Parent [c]	Paternal Parent [c]	*S*-genotype	SI/SC prediction	SI/SC phenotype
27 2(17)	UF	Surefire	$S_{13'}S_aS_dS_e$	SC	SC
27 2(37)	UF	Surefire	$S_{13'}S_aS_dS_e$	SC	SC
27 2(58)	UF	Surefire	$S_{13'}S_aS_dS_e$	SC	SC
27 3(1)	UF	Surefire	$S_{13'}S_aS_dS_e$	SC	SC
27 3(24)	UF	Surefire	$S_{13'}S_aS_dS_e$	SC	SC
27 3(42)	UF	Surefire	$S_{13'}S_aS_dS_e$	SC	SC
27 3(63)	UF	Surefire	$S_{13'}S_aS_dS_e$	SC	SC
27 4(43)	UF	Surefire	$S_{13'}S_aS_dS_e$	SC	SC
27 2(45)	UF	Surefire	$S_4S_{13'}S_aS_e$	SC	SC
27 3(16)	UF	Surefire	$S_4S_{13'}S_aS_e$	SC	SC
27 3(65)	UF	Surefire	$S_4S_{13'}S_aS_e$	SC	SC
27 2(61)	UF	Surefire	$S_4S_{13'}S_aS_d$	SC	SC
27 3(20)	UF	Surefire	$S_4S_{13'}S_aS_d$	SC	SC
27 2(23)	UF	Surefire	$S_{13'}S_aS_eS_x$	SC	SC
27 2(32)	UF	Surefire	$S_{13'}S_aS_eS_x$	SC	SC
27 2(43)	UF	Surefire	$S_{13'}S_aS_eS_x$	SC	SC
27 3(46)	UF	Surefire	$S_{13'}S_aS_eS_x$	SC	SC
27 3(48)	UF	Surefire	$S_{13'}S_aS_dS_x$	SC	SC
27 4(10)	UF	Surefire	$S_{13'}S_aS_dS_x$	SC	SC
27 2(24)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 2(48)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 2(51)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 2(57)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 2(65)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 3(2)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC

(Continued)

Plant ID	Maternal Parent [c]	Paternal Parent [c]	*S*-genotype	SI/SC prediction	SI/SC phenotype
27 3(28)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 3(41)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 4(7)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 4(31)	UF	Surefire	$S_4S_{13'}S_aS_x$	SC	SC
27 13(32)	UF	RS	$S_{13'}S_aS_eS_x$	SC	SC
27 13(51)	UF	RS	$S_{13'}S_dS_eS_x$	SC	SC
27 13(59)	UF	RS	$S_{13'}S_dS_eS_x$	SC	SC
27 13(65)	UF	RS	$S_{13'}S_dS_eS_x$	SC	SC
27e 2(27)	UF	RS	$S_{13'}S_dS_eS_x$	SC	SC
27 13(45)	UF	RS	$S_6S_{13'}S_eS_x$	SC	SC
27 13(56)	UF	RS	$S_6S_{13'}S_eS_x$	SC	SC
27 13(57)	UF	RS	$S_4S_{13'}S_eS_x$	SC	SC
27e 2(28)	UF	RS	$S_{13'}S_bS_dS_e$	SC	SC
27 13(37)	UF	RS	$S_4S_6S_bS_d$	SI	SI
27 13(42)	UF	RS	$S_4S_6S_bS_d$	SI	SI
27 13(61)	UF	RS	$S_4S_6S_bS_d$	SI	SI
27 13(36)	UF	RS	$S_4S_6S_bS_x$	SI	SI
27 9(12)	UF	III 18(12)	$S_4S_{13'}S_fS_x{}^a$	SC	SC
27 9(14)	UF	III 18(12)	$S_{13'}S_aS_eS_x$	SC	SC
27 9(15)	UF	III 18(12)	$S_{13'}S_dS_eS_f{}^a$	SC	SC
27 9(23)	UF	III 18(12)	$S_{13'}S_dS_eS_f{}^a$	SC	SC
27 9(25)	UF	III 18(12)	$S_4S_{13'}S_dS_f{}^a$	SC	SC
27 9(27)	UF	III 18(12)	$S_{13'}S_dS_eS_f{}^a$	SC	SC
27 8(58)	Mont	Jubileum	$S_6S_eS_gS_x{}^b$	SC	SC
27 8(59)	Mont	Jubileum	$S_{13'}S_aS_aS_g{}^b$	SC	SC
27 8(62)	Mont	Jubileum	$S_{13'}S_aS_eS_x$	SC	SC
27 12(51)	UF	Mont	$S_aS_dS_eS_x$	SC	SC
27 23(19)	UF	Mont	$S_{13'}S_aS_dS_x$	SC	SC

(Continued)

Plant ID	Maternal Parent	Paternal Parent	*S*-genotype	SI/SC prediction	SI/SC phenotype
27 23(29)	UF	Mont	$S_{13'}S_dS_eS_x$	SC	SC
27 23(35)	UF	Mont	$S_{13'}S_dS_eS_x$	SC	SC
27 12(54)	UF	Mont	$S_{13'}S_dS_eS_x$	SC	SI
27 12(50)	UF	Mont	$S_4S_6S_aS_e$	SC	SC
27 23(22)	UF	Mont	$S_4S_6S_{13'}S_d$	SC	SC
27 23(16)	UF	Mont	$S_4S_6S_aS_x$	SI	SI
27 23(42)	UF	Mont	$S_4S_6S_aS_x$	SI	SI

[a] S_f represents a unique *S*-haplotype in III 18(12). The functionality of S_f is unknown.

[b] S_g represents a unique *S*-haplotype in Jubileum. The functionality of S_g is unknown.

[c] UF = Újfehértói fürtös; RS = Rheinische Schattenmorelle; Mont = Montmorency

Table B.2: Thirteen sour cherry crosses used to test the validity of the hypothesis for the genetic control of SI and SC in sour cherry. The compatibility or incompatibility of each cross was predicted based on the *S*-genotype of the parents.

Maternal Parent		Paternal Parent		Compatible (C) or Incompatible (I)	
Plant ID	*S*-genotype	Plant ID	*S*-genotype	Prediction	Phenotype
25 2(22)	$S_4S_6S_bS_x$	27 2(24)	$S_4S_{13'}S_aS_x$	C	C
27 2(24)	$S_4S_aS_cS_x$	25 2(22)	$S_4S_6S_bS_x$	C	C
25 2(22)	$S_4S_6S_bS_x$	27 2(65)	$S_4S_{13'}S_aS_x$	C	C
27 2(65)	$S_4S_{13'}S_aS_x$	25 2(22)	$S_4S_6S_bS_x$	C	C
25 3(35)	$S_4S_6S_aS_b$	27 2(24)	$S_4S_{13'}S_aS_x$	C	C
27 2(24)	$S_4S_{13'}S_aS_x$	25 3(35)	$S_4S_6S_aS_b$	C	C
25 3(35)	$S_4S_6S_aS_b$	27 2(65)	$S_4S_{13'}S_aS_x$	C	C
27 2(65)	$S_4S_{13'}S_aS_x$	25 3(35)	$S_4S_6S_aS_b$	C	C
25 3(4)	$S_4S_{13'}S_aS_x$	27 13(61)	$S_4S_6S_bS_x$	C	C
27 13(61)	$S_4S_6S_bS_x$	25 3(4)	$S_{13'}S_aS_4S_x$	C	C
27 13(61)	$S_4S_6S_bS_x$	25 3(28)	$S_4S_6S_bS_x$	I	I
27 13(45)	$S_6S_{13'}S_eS_x$	25 3(28)	$S_4S_6S_bS_x$	C	C
27 13(57)	$S_4S_aS_{13'}S_e$	25 3(28)	$S_bS_4S_6S_x$	C	C

Table B.3: The *S*-genotype and SI or SC phenotype for 81 progeny in the Rheinische Schattenmorelle (RS) x Érdi Bőtermő (EB) population.

Plant ID	*S*-genotype	SI/SC Prediction [a]	SI/SC Phenotype [b]
25 2(7)	$S_4S_6S_aS_x$	SI or SC	SI
25 2(15)	$S_4S_6S_aS_x$	SI or SC	SC
25 2(23)	$S_4S_6S_aS_x$	SI or SC	SI
25 2(36)	$S_4S_6S_aS_x$	SI or SC	SI
25 2(38)	$S_4S_6S_aS_x$	SI or SC	-
25 2(44)	$S_4S_6S_aS_x$	SI or SC	SC
25 2(50)	$S_4S_6S_aS_x$	SI or SC	SI
25 2(52)	$S_4S_6S_aS_x$	SI or SC	SC
25 2(62)	$S_4S_6S_aS_x$	SI or SC	SI
25 2(65)	$S_4S_6S_aS_x$	SI or SC	SI
25 3(07)	$S_4S_6S_aS_x$	SI or SC	-
25 3(21)	$S_4S_6S_aS_x$	SI or SC	SC
25 3(34)	$S_4S_6S_aS_x$	SI or SC	SC
25 2(3)	$S_4S_aS_bS_x$	SI or SC	SC
25 2(17)	$S_4S_aS_bS_x$	SI or SC	SI
25 2(39)	$S_6S_aS_xS_x$	SC	-
25 2(53)	$S_6S_aS_xS_x$	SC	SI [c]
25 3(29)	$S_6S_aS_xS_x$	SC	SC
25 2(33)	$S_aS_bS_xS_x$	SC	-
25 2(40)	$S_aS_bS_xS_x$	SC	-
25 2(28)	$S_{13'}S_aS_bS_x$	SC	-
25 2(18)	$S_4S_{13'}S_aS_b$	SC	SC
25 2(32)	$S_4S_{13'}S_aS_b$	SC	SC
25 2(46)	$S_4S_{13'}S_aS_b$	SC	-
25 2(58)	$S_4S_{13'}S_aS_b$	SC	SC
25 3(02)	$S_4S_{13'}S_aS_b$	SC	-
25 3(06)	$S_4S_{13'}S_aS_b$	SC	-
25 3(08)	$S_4S_{13'}S_aS_b$	SC	SC
25 2(16)	$S_{13'}S_aS_xS_x$	SC	SC
25 2(8)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(10)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(12)	$S_4S_{13'}S_aS_x$	SC	SC

Plant ID	*S*-genotype	SI/SC Prediction [a]	SI/SC Phenotype [b]
25 2(14)	$S_4S_{13'}S_aS_x$	SC	-
25 2(20)	$S_4S_{13'}S_aS_x$	SC	-
25 2(25)	$S_4S_{13'}S_aS_x$	SC	-
25 2(29)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(35)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(43)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(47)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(48)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(51)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(55)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(66)	$S_4S_{13'}S_aS_x$	SC	SC
25 3(04)	$S_4S_{13'}S_aS_x$	SC	SC
25 3(09)	$S_4S_{13'}S_aS_x$	SC	SC
25 3(14)	$S_4S_{13'}S_aS_x$	SC	SC
25 3(16)	$S_4S_{13'}S_aS_x$	SC	SC
25 3(31)	$S_4S_{13'}S_aS_x$	SC	SC
25 3(37)	$S_4S_{13'}S_aS_x$	SC	SC
25 2(05)	$S_4S_6S_{13'}S_a$	SC	SC
25 2(11)	$S_4S_6S_{13'}S_a$	SC	SC
25 2(49)	$S_4S_6S_{13'}S_a$	SC	SC
25 2(56)	$S_4S_6S_{13'}S_a$	SC	SC
25 2(63)	$S_4S_6S_{13'}S_a$	SC	SC
25 3(03)	$S_4S_6S_{13'}S_a$	SC	SC
25 3(05)	$S_4S_6S_{13'}S_a$	SC	SC
25 3(20)	$S_4S_6S_{13'}S_a$	SC	SC
25 2(30)	$S_6S_{13'}S_aS_x$	SC	-
25 2(06)	$S_4S_6S_aS_b$	SI	SI
25 2(37)	$S_4S_6S_aS_b$	SI	SI
25 2(42)	$S_4S_6S_aS_b$	SI	SI
25 3(10)	$S_4S_6S_aS_b$	SI	-
25 3(32)	$S_4S_6S_aS_b$	SI	SI
25 3(35)	$S_4S_6S_aS_b$	SI	SI
25 2(13)	$S_4S_6S_bS_x$	SI	SI
25 2(22)	$S_4S_6S_bS_x$	SI	SI
25 2(41)	$S_4S_6S_bS_x$	SI	SI
25 2(45)	$S_4S_6S_bS_x$	SI	SI

Plant ID	*S*-genotype	SI/SC Prediction [a]	SI/SC Phenotype [b]
25 2(54)	$S_4S_6S_bS_x$	SI	SI
25 2(60)	$S_4S_6S_bS_x$	SI	SI
25 2(64)	$S_4S_6S_bS_x$	SI	SI
25 3(25)	$S_4S_6S_bS_x$	SI	-
25 3(28)	$S_4S_6S_bS_x$	SI	SI
25 2(2)	$S_4S_{13}S_bS_x$	SI	-
25 2(59)	$S_4S_{13}S_bS_x$	SI	SI
25 3(13)	$S_4S_{13}S_bS_x$	SI	SC [c]
25 3(18)	$S_4S_{13}S_bS_x$	SI	-
25 3(22)	$S_4S_{13}S_bS_x$	SI	SI
25 2(19)	$S_4S_6S_{13}S_x$	SI	SC [c]
25 2(27)	$S_4S_6S_{13}S_x$	SI	SI
25 3(24)	$S_4S_6S_{13}S_x$	SI	-

[a] See Figure B.1 for explanation of how the SI or SC predictions were made

[b] The SI or SC phenotype was determined through observation of pollen tube growth down self-pollinated styles. "-" denotes individuals for which the SI or SC phenotype could not be determined.

[c] The SI or SC phenotypes of these three individuals were incorrectly predicted.

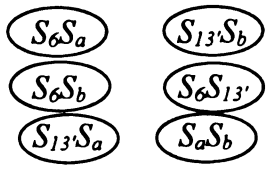
Chromosome pairing in RS

S_6	S_a
$S_{13'}$	S_b

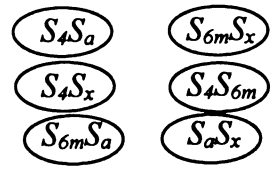
Chromosome pairing in EB

S_4	S_a
S_{6m}	S_x

Possible RS Gametes



Possible EB Gametes



Note: Pollen containing S_{6m} are incompatible with RS styles

Punnett Square

EB gametes

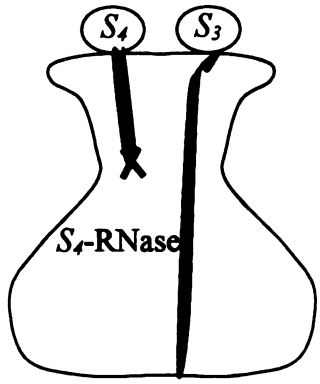
RS gametes	S_4S_a	S_4S_x	S_aS_x
S_6S_a	$S_4S_6S_aS_a$ SC	$S_4S_6S_aS_x$ SI	$S_6S_aS_aS_x$ SC
S_6S_b	$S_4S_6S_aS_b$ SI	$S_4S_6S_bS_x$ SI	$S_6S_bS_aS_x$ SI
$S_{13'}S_a$	$S_4S_{13'}S_aS_a$ SC	$S_4S_{13'}S_aS_x$ SC	$S_{13'}S_aS_aS_x$ SC
$S_{13'}S_b$	$S_4S_{13'}S_aS_b$ SC	$S_4S_{13'}S_bS_x$ SI	$S_{13'}S_bS_aS_x$ SC
$S_6S_{13'}$	$S_4S_6S_{13'}S_a$ SC	$S_4S_6S_{13'}S_x$ SI	$S_6S_{13'}S_aS_x$ SC
S_aS_b	$S_4S_aS_aS_b$ SC	$S_4S_aS_bS_x$ SI	$S_aS_aS_bS_x$ SC

Note: When progeny were *S*-genotyped, it was not possible to determine dosage. Thus, $S_4S_6S_aS_a$ appeared the same as $S_4S_6S_aS_x$

Figure B.1: Graphic explanation of the expected *S*-genotypes and SI or SC phenotypes of progeny in the Rheinische Schattenmorelle (RS) x Érdi Bőtermő (EB) population shown in Table B.3.

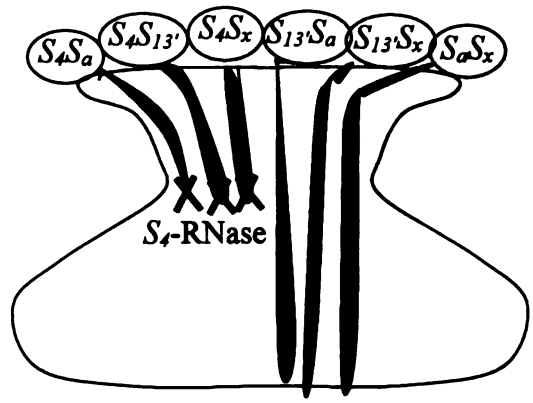
Figure B.2: Schematic representation of the reciprocal inter-specific crosses between sweet and sour cherry analyzed in Chapter 4. All possible pollen types are shown on the stigma. Pollen tube growth either stops half-way down the style (if the pollen tube encounters a cognate *S*-RNase that is functional) or grows to the bottom of the pistil and fertilizes an egg (if the pollen tube does not encounter its functional cognate *S*-RNase). The only *S*-RNases shown in the styles are those that match an *S*-haplotype in the pollen. The non-functional S_{6m} and S_{6c}-RNases from EB and Cigany 59, respectively, are denoted by large X's through the *S*-RNase. EF = Emperor Francis; UF = Újfehértói fürtös; EB = Érdi Bõtermõ; RS = Rheinische Schattenmorelle; Mont = Montmorency.

Surefire ($S_4S_{13'}S_aS_x$) x EF (S_3S_4)



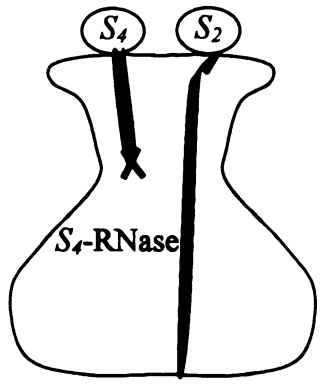
Concl: Surefire *S_4-RNase* is functional

EF (S_3S_4) x Surefire ($S_4S_{13'}S_aS_x$)



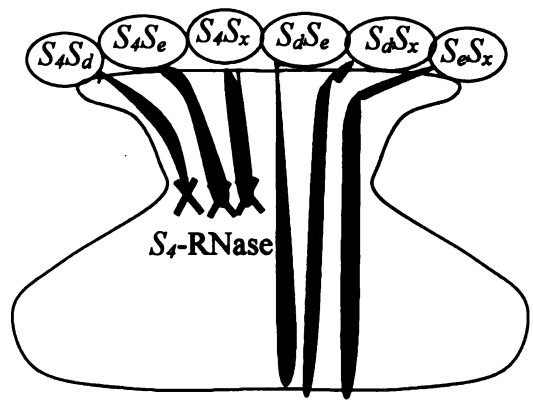
Concl: Surefire *pollen-S_4* gene is functional

UF ($S_4S_dS_eS_x$) x Schmidt (S_2S_4)



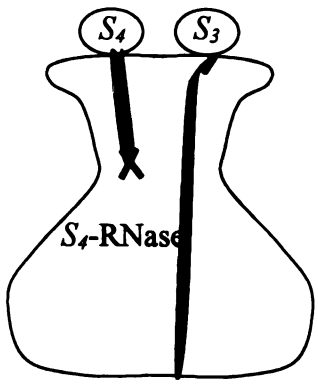
Concl: UF *S_4-RNase* is functional

EF (S_3S_4) x UF ($S_4S_dS_eS_x$)



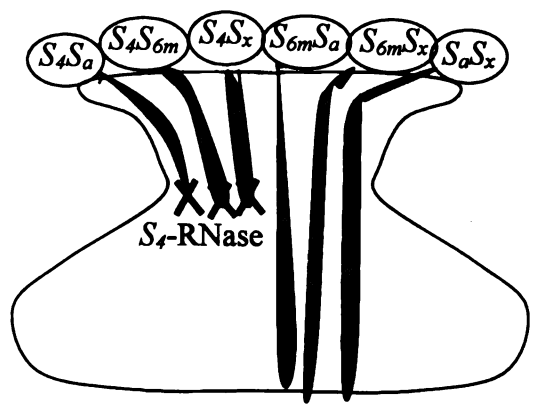
Concl: UF *pollen-S_4* gene is functional

EB ($S_4S_{6m}S_aS_x$) x EF (S_3S_4)



Concl: EB *S_4-RNase* is functional

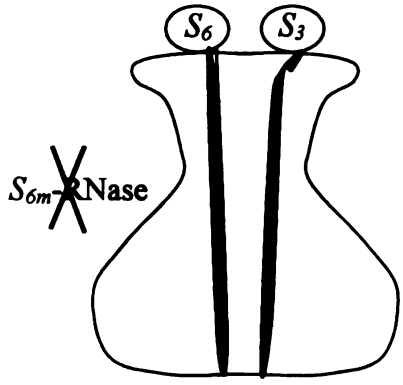
EF (S_3S_4) x EB ($S_4S_{6m}S_aS_x$)



Concl: EB *pollen-S_4* gene is functional

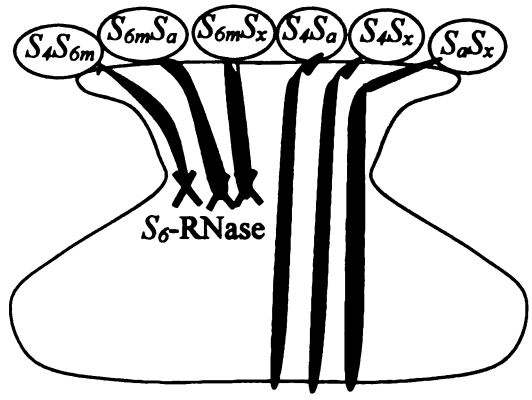
(Continued)

EB ($S_4S_{6m}S_aS_x$) x Gold (S_3S_6)



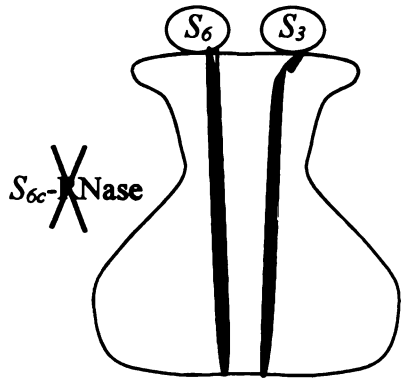
Concl: EB *S_{6m}-RNase* is not functional

Gold (S_3S_6) x EB ($S_4S_{6m}S_aS_x$)



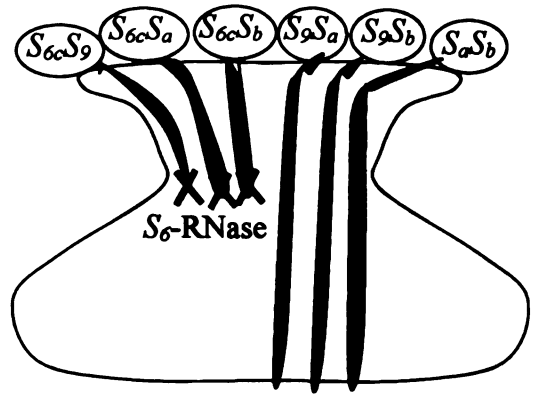
Concl: EB *pollen-S_{6m}* gene is functional

Cigany 59 ($S_{6c}S_9S_aS_b$) x Gold (S_3S_6)



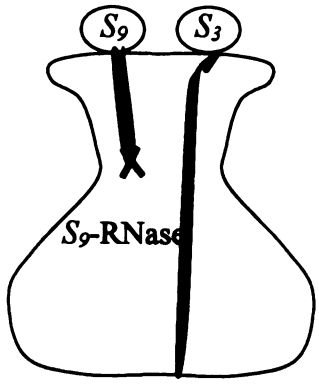
Concl: Cigany 59 *S_{6c}-RNase* is not functional

Gold (S_3S_6) x Cigany 59 ($S_{6c}S_9S_aS_b$)



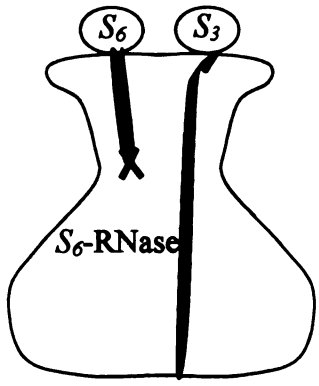
Concl: Cigany 59 *pollen-S_{6c}* gene is functional

Cigany 59 ($S_{6c}S_9S_aS_b$) x Chelan (S_3S_9)



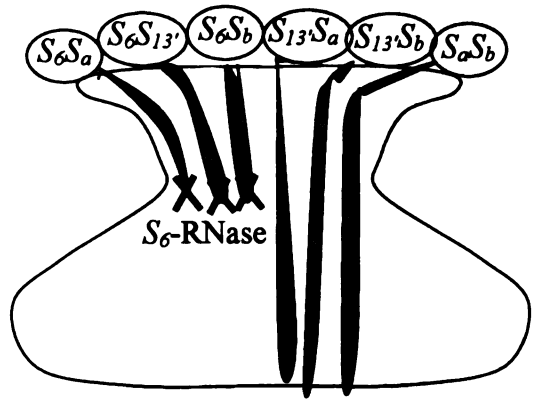
Concl: Cigany 59 *S_9-RNase* is functional

RS ($S_6S_{13'}S_aS_b$) x Gold (S_3S_6)



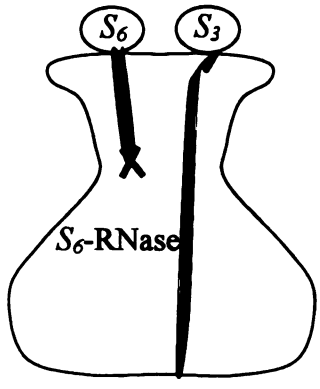
Concl: RS *S_6-RNase* is functional

Gold (S_3S_6) x RS ($S_6S_{13'}S_aS_b$)



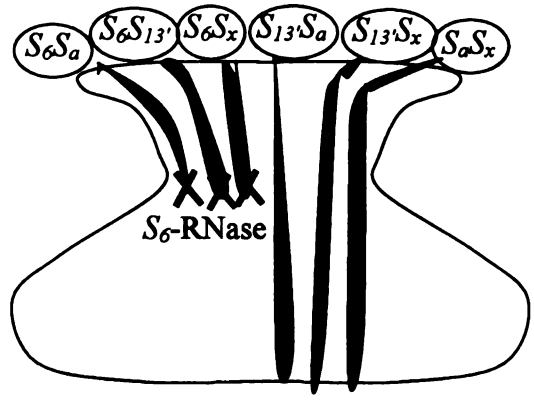
Concl: RS *pollen-S_6* gene is functional

Mont ($S_6S_{13'}S_aS_x$) x Gold (S_3S_6)



Concl: Mont *S_6-RNase* is functional

Gold (S_3S_6) x Mont ($S_6S_{13'}S_aS_x$)



Concl: Mont *pollen-S_6* gene is functional

Rheinische Schattenmorelle ($S_6S_{13'}S_aS_b$)

Functionality of *S*-haplotypes

S_6	Functional
$S_{13'}$	Non-Functional. Probably pollen-part mutant
S_a	Non-Functional. Probably pollen-part mutant
S_b	Functional

Chromosome Pairing

S_6	S_a
$S_{13'}$	S_b

Fragment sizes of *S-RNases*

	S_6	$S_{13'}$	S_a	S_b
*Hin*dIII	5.8 kb	4.6 kb	6.4 kb	5.1 kb
*Xba*I	5.5 kb	9.4 kb	2.4 kb	5 kb
Pruc2/PCE	300 bp	620 bp	730 bp	550 bp

Gamete types

S_6S_a	$S_{13'}S_b$
S_6S_b	$S_6S_{13'}$ *
$S_{13'}S_a$	S_aS_b *

* Requires multivalent formation

Self-Pollination

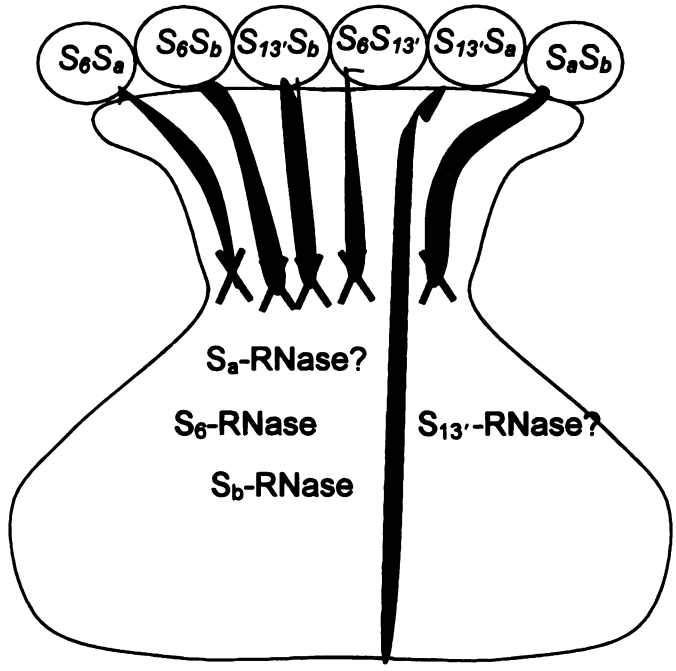


Figure B.3: Summary of the *S*-haplotypes in Rheinische Schattenmorelle

Érdi Bõtermõ ($S_4S_{6m}S_a$)

Functionality of S-haplotypes

S_4	Functional
S_{6m}	Non-Functional. S-RNase mutant. 2600 bp insertion in promoter
S_a	Non-Functional. Probably pollen-part mutant

Chromosome Pairing

S_4	S_a
S_{6m}	S_x

Fragment sizes of *S-RNases*

	S_4	S_{6m}	S_a
*Hin*dIII	5.6 + 6.1 kb	9 kb	6.4 kb
*Xba*I	8.8 kb	5.5 kb	2.4 kb
Pruc2/PCE	850 bp	300 bp	730 bp

Gamete types

S_4S_a	$S_{6m}S_x$
S_4S_x	S_4S_{6m} *
$S_{6m}S_a$	S_aS_x *

* Requires multivalent formation

Self-Pollination

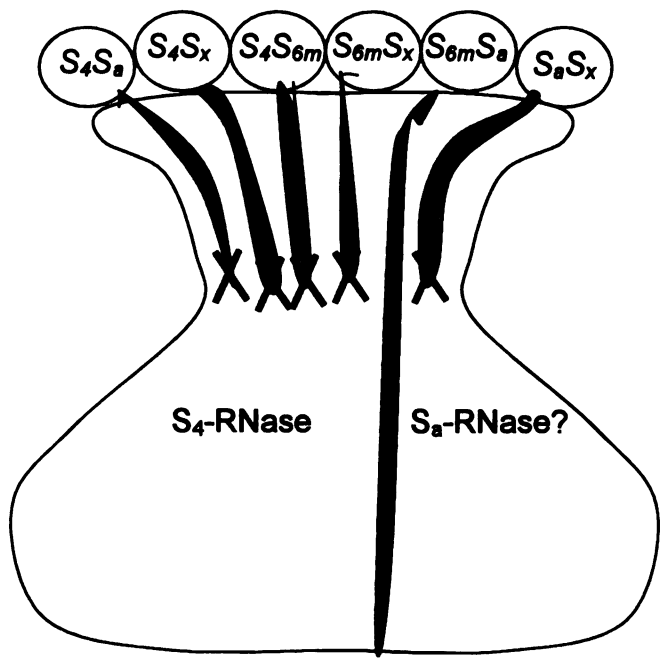


Figure B.4: Summary of the *S*-haplotypes in Érdi Bõtermõ

Cigány 59 ($S_{6c}S_9S_aS_b$)

Functionality of S-haplotypes

S_{6c} Non-Functional. *S-RNase* mutant

S_9 Functional

S_a Non-Functional. Probably pollen-part mutant

S_b Functional

Chromosome Pairing

S_{6c}	S_a
S_9	S_b

Fragment sizes of *S-RNases*

	S_{6c}	S_9	S_a	S_b
*Hin*dIII	5.8 kb	3.1 + 4.0 kb	6.4 kb	5.1 kb
*Xba*I	5.5 kb	15 kb	2.4 kb	5 kb
Pruc2/PCE	300 bp	550 bp	730 bp	550 bp

Gamete types

$S_{6c}S_a$	S_9S_b
$S_{6c}S_b$	$S_{6c}S_9$ *
S_9S_a	S_aS_b *

* Requires multivalent formation

Self-Pollination

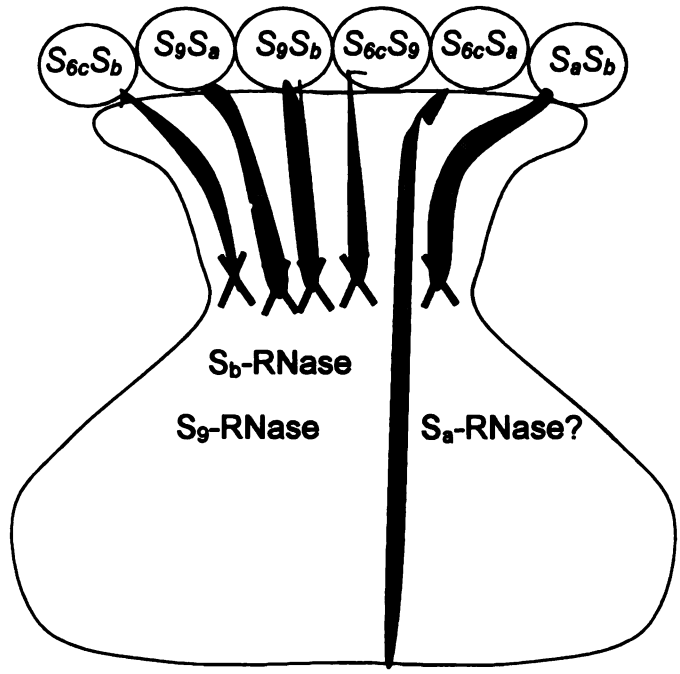


Figure B.5: Summary of the *S*-haplotypes in Cigány 59

Surefire ($S_4S_{13'}S_a$)

Functionality of S-haplotypes

S_4 Functional

$S_{13'}$ Non-Functional. Probably pollen-part mutant

S_a Non-Functional. Probably pollen-part mutant

Chrom40osome Pairing

S_4	S_a
$S_{13'}$	S_x

Fragment sizes of *S-RNases*

	S_4	$S_{13'}$	S_a
*Hin*dIII	5.6 + 6.1 kb	4.6 kb	6.4 kb
*Xba*I	8.8 kb	9.4 kb	2.4 kb
Pruc2/PCE	850 bp	620 bp	730 bp

Gamete types

S_4S_a	$S_{13'}S_x$
S_4S_x	$S_4S_{13'}$*
$S_{13'}S_a$	S_aS_x*

* Requires multivalent formation

Self-Pollination

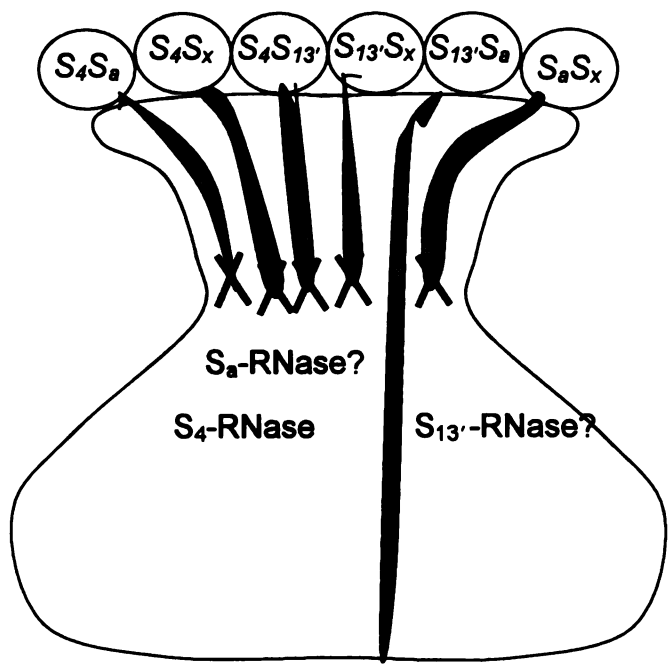


Figure B.6: Summary of the *S*-haplotypes in Surefire

Montmorency ($S_6S_aS_{13'}S_x$)

Functionality of S-haplotypes

S_6	Functional
$S_{13'}$	Non-Functional. Probably pollen-part mutant
S_a	Non-Functional. Probably pollen-part mutant

Chromosome Pairing

S_6	S_a
$S_{13'}$	S_x

Fragment sizes of *S-RNases*

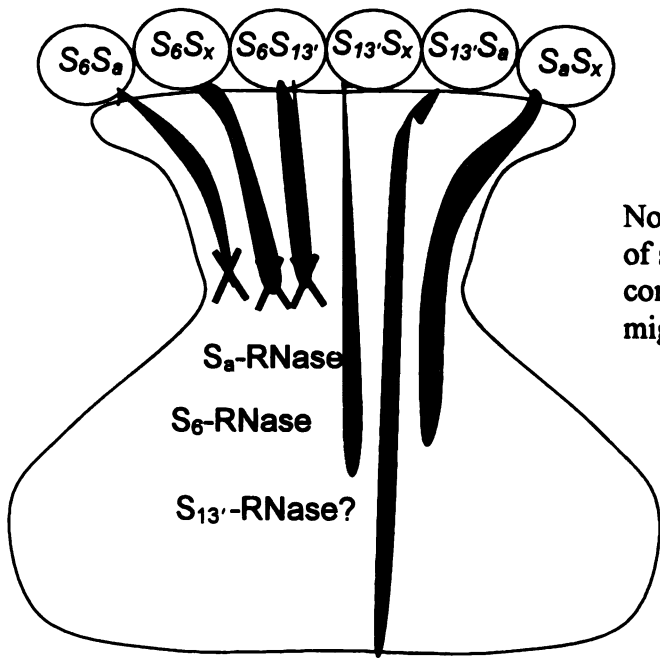
	S_6	$S_{13'}$	S_a
*Hin*dIII	5.8 kb	4.6 kb	6.4 kb
*Xba*I	5.5 kb	9.4 kb	2.4 kb
Pruc2/PCE	300 bp	620 bp	730 bp

Gamete types

S_6S_a	$S_{13'}S_x$
S_6S_x	$S_6S_{13'}$ *
$S_{13'}S_a$	S_aS_x *

* Requires multivalent formation

Self-Pollination



Note: S_x appears to be capable of self-fertilization, but pollen containing the pollen S_x-product might be less competitive

Figure B.7: Summary of the *S*-haplotypes in Montmorency

Újfehértói fürtös ($S_4S_dS_eS_x$)

Functionality of *S*-haplotypes

4	Functional
S_d	Non-Functional. Possibly similar to S_a
S_e	Non-Functional. Pollen-part mutant of S_1

Chromosome Pairing

S_4	S_d
S_e	S_x

Fragment sizes of *S-RNases*

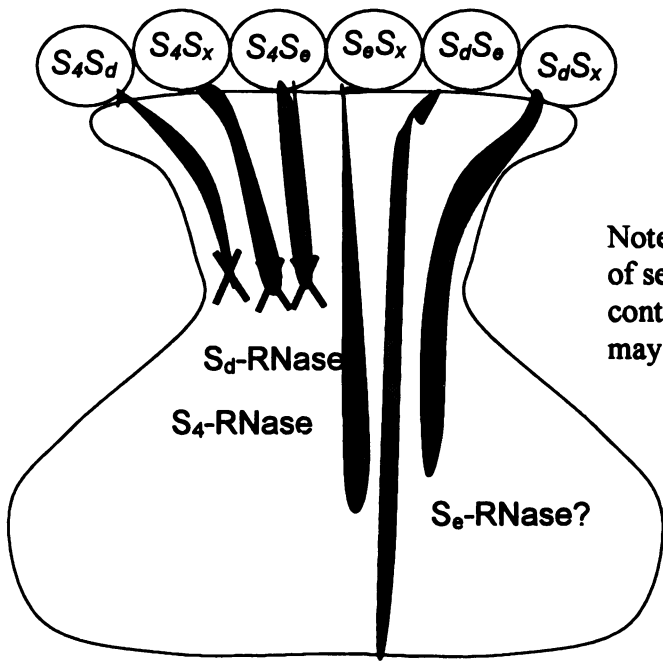
	S_4	S_d	S_e
*Hin*dIII	5.6+ 6.1 kb	6.2 kb	9.6 kb
*Xba*I	8.8 kb	2.4 kb	? kb
Pruc2/PCE	850 bp	730 bp	620 bp

Gamete types

S_4S_d	S_eS_x
S_4S_x	S_4S_e *
S_dS_e	S_dS_x *

* Requires multivalent formation

Self-Pollination



Note: S_x appears to be capable of self-fertilization, but pollen containing the pollen S_x-product may be less competitive

Figure B.8: Summary of the *S*-haplotypes in Újfehértói fürtös