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Review of the molecular background of self-incompatibility in rosaceous fruit trees

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Summary: This review gives a presentation of the gametophytic self-incompatibility system in the roscaeous fruit trees. Studies to discover the pistil (*S*-ribonucleases) and pollen-part components (F-box molecules) are summarized and models for the self-incompatibility reactions as well as their molecular background are discussed. We describe how mutations within the *S*-RNase or F-box genes can contribute to the transition from self-incompatibility to the self-compatible phenotype in many fruit tree crops. The current state of the arts is compared to the information obtained in other plant species possessing similar incompatibility system.

Key words: F-box, pistil, pollen, *Rosaceae*, self-incompatibility, *S*-genotype, *S*-RNase

Types of the plant self-incompatibility system

Many flowering plants bear the male and female organs in close proximity within the same flower. The physical and temporal development of the flower is such that self-fertilization would occur in the absence of specific mechanisms to prevent it. Self-incompatibility (SI) systems, together with other devices promoting cross-pollination such as dichogamy, dioecism, and various specializations of flower structure, regulate the breeding system by ensuring a greater or lesser amount of outcrossing in a population (*Heslop-Harrison*, 1975). SI has been a favourite topic for botanists and geneticists since *Darwin* (1877) first discussed the phenomenon and suggested the idea of its central significance during the evolution of flowering plants. Within the last two decades, scientists have been able to complement Darwin's genetic observations with molecular and biochemical analyses which have significantly contributed to the elucidation of the complex series of interactions occurring at the pollen-stigma interface (*Silva & Goring*, 2001).

A generalized definition of self-incompatibility is "the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination" (*de Nettancourt*, 1977). While many flowering plants rely on SI to ensure genetic variability, not all species depend upon the same system. There are two main types of self-incompatibility: heteromorphic and homomorphic. Heteromorphically self-incompatible species produce morphologically distinct flowers that provide a physical barrier to pollination within each flower but allows outcrossing. The flowers of homomorphic species have the reproductive organs in close proximity to each other. It is very widely distributed across a number of taxa and is divided into two subgroups, gametophytic and sporophytic (*Clarke & Newbigin*, 1993).

Homomorphic SI is estimated to be present in more than half of all species of angiosperms (*de Nettancourt*, 1977). Of the 383 families of angiosperms, self-incompatibility has been described in 81 families. Among them, 15 families have well-described gametophytic SI, 39 definitely have SI but of an undefined type, and 21 may have SI although it has not been confirmed yet (*Charlesworth*, 1985). Sporophytic SI has been described in 6 families (*Gibbs & Ferguson*, 1987). It is thought that incompatibility contributed greatly to the success of angiosperms very early in their ancestry, which explains its wide occurrence among wild species. For domesticated species, the situation is slightly different as humans have selected for the ability to set fruit (*Wiersma*, 2003).

Gametophytic self-incompatibility (GSI) is employed by a number of families, however, only a few have been studied at molecular level. The most extensively studied families are the *Solanaceae*, *Rosaceae*, *Scrophulariaceae* and *Papaveraceae*. In most families, GSI is controlled by a single locus (*S*-locus, termed after the word "sterility") with multiple alleles. However, there are more complex systems involving several gene loci, for example, some grass species have two loci (*Lundqvist*, 1956) or *Beta vulgaris* has four loci (*Larsen*, 1977). Sporophytic self-incompatibility (SSI) is not as widespread as GSI and it is largely studied in the *Brassicaceae* and *Asteraceae*.

In gametophytic SI, the phenotype of the pollen is determined by its own haploid genotype. In the *Rosaceae*

family, GSI is controlled by the single, polymorphic *S*-locus. Fertilization is prevented when the *S*-allele expressed by the haploid pollen grain matches one of the *S*-alleles expressed in the pistil. Pollen grains from the S_1S_2 anther are incompatible with the S_1S_2 pistil. If two different cultivars have identical *S*-genotypes, it presents an incompatible combination in each direction (*Kozma* et al., 2003; *Nyéki & Szabó*, 1995). These cultivars are mutually self-incompatible, in other terms cross- or inter-incompatible. When only one of the pollen alleles, S_1, is shared with those in the pistil, then half of the pollen grains, the S_3 pollen, are compatible on the S_1S_2 pistil, so presents a semi-compatible combination. Pollen grains from the S_3S_4 anther bear different *S*-alleles than the S_1S_2 pistil, so they are fully compatible (*Figure 1A*).

In sporophytic SI, the phenotype of the pollen is determined by the diploid genome of the parental plant. Thus, when one of the *S*-alleles in the pollen parent matches that of the pistil, pollen germination is arrested at the stigma surface. Pollen grains of both the S_1S_2 or S_1S_3 anthers are rejected in the S_1S_2 pistils due to the matching alleles (*Figure 1B*). However, pollen grains from the S_1S_2 anther are fully compatible on an S_3S_4 pistil, and fertilization occurs. *S*-alleles in the pollen grain may show dominance relationships with one another, and in this case the *S*-phenotype of the pollen depends on the possible interaction between its parental alleles (*Thompson & Kirch*, 1992). It is a well-known phenomenon in *Brassica* species as well as in *Corylus avellana*, the only fruit species known to have sporophytic self-incompatibility. Recent studies have revealed a lot from the molecular events behind the SSI of *Brassicaceae*, however, details of sporophytic SI in hazelnut are still completely unknown.

Molecular analyses of self-incompatibility systems have focused on identifying and characterizing the pollen and pistil components of the self-incompatible response as well as other proteins and events that lead to pollen rejection. In this review, we discuss the molecular biology of the *S*-RNase based GSI system in the *Rosaceae* family.

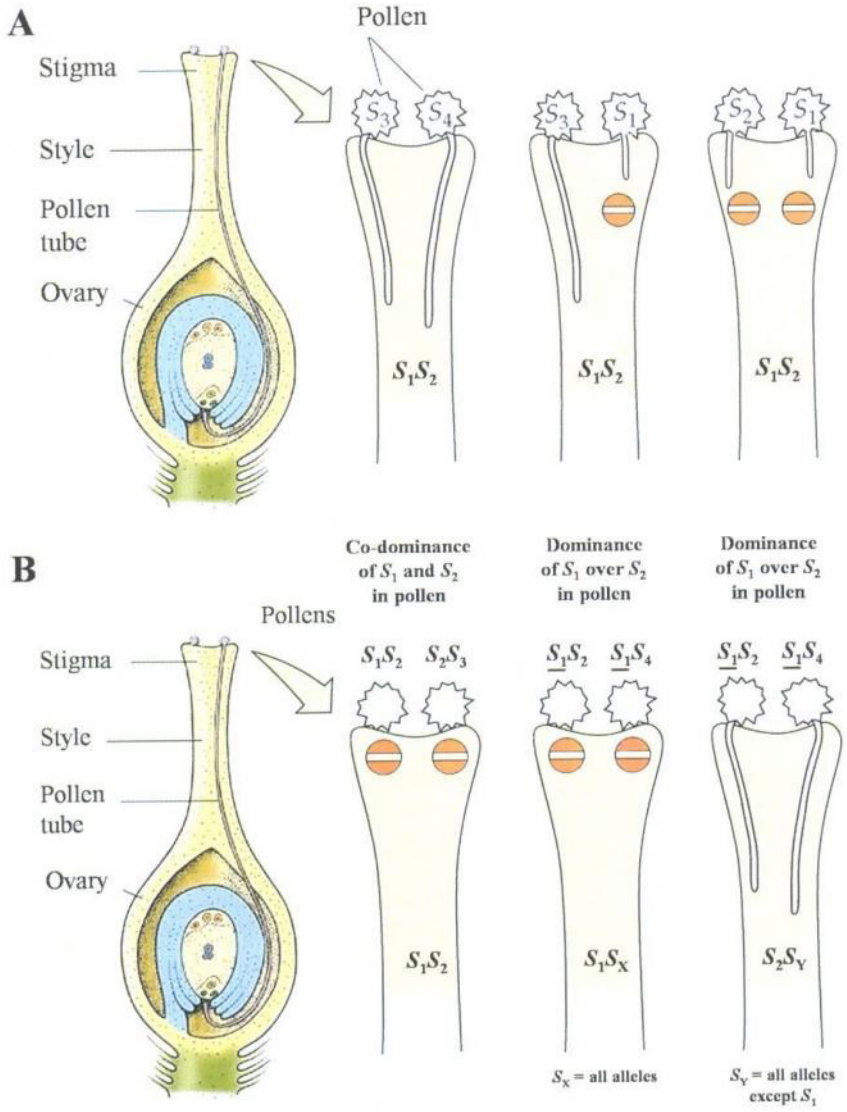

Figure 1 Genetic control of the two main self-incompatibility systems in higher plants. (A) Gametophytic self-incompatibility. The incompatibility phenotype of a pollen grain is determined by its own haploid genotype and the *S*-alleles are generally co-dominant. If the *S*-allele expressed in the pollen is identical with either of the *S*-alleles expressed in the pistil, pollen tube growth is inhibited in the upper third part of the style. (B) Sporophytic self-incompatibility. The incompatibility phenotype of a pollen grain depends on the genotype of the pollen parent. In contrast to the gametophytic system, the *S*-alleles are not always co-dominant. Fertilization is prevented if any (co-dominant manner) or a certain one (dominant manner) of the two *S*-alleles of the pollen-producing plant is also expressed in the pistils of plants to be fertilized. Germination of the pollen grain is inhibited at the stigmatic surface. Modified after *Thompson & Kirch* (1992)

The pistil component: an astonishing function for plant ribonucleases?

Ribonucleic acid was discovered at the end of the 19th century. Ribonuclease (RNase) is an expression used to denominate enzymes that split RNA molecules in a highly substrate-specific manner. These RNA-degrading enzymes were discovered by *Jones* in 1920. Also a

bovine pancreatic RNase was the first enzyme to be chemically sequenced (*Blackburn & Moore*, 1982). Later it was shown that this RNase is a member of superfamily of enzymes with roles extending far beyond the digestion of dietary RNA, and exhibit several physiological activities, including neurotoxic or antitumor effects (*D'Alessio*, 1993).

First report about RNases in plant style described that their activity varied greatly among species (*Schrauwen & Linskens*, 1972). A discovery, which gave further background for the hypothesis that RNases may have more diverse functions in plants was made in 1989 by Adrienne E. Clarke's research team at the University of Melbourne (*McClure* et al., 1989). They were the first who clarified that the basic *S*-glycoproteins associated with gametophytic self-incompatibility (GSI) in *Nicotiana alata* possessed inherent ribonuclease activity. We must mention that an antigenic glycoprotein associated with the self-incompatibility genotype (Antigen *S*) was isolated from *Prunus avium* styles and partially characterized in professor Clarke's laboratory at the beginning of the 1980s (*Mau* et al., 1982). This component had substantial (16.3%) carbohydrate content, a molecular weight of 37–39 kDa and a high isoelectric point (pI 10.6). Williams et al. (1982) have clarified that the Antigen *S* is a potent inhibitor of *in vitro* self-pollen tube growth, suggesting that it represents the *S*-gene product. They have got further inspiration from the results of their subsequent works: *Anderson* et al. (1986) sequenced S_2-allele of *Nicotiana alata*, which showed precise homology with other *N. alata* and *Lycopersicon peruvianum* *S*-glycoproteins as well as with fungal ribonucleases, RNase T_2 (*Kawata* et al., 1988) and Rh (*Horiuchi* et al., 1988). *S*-glycoproteins were purified from style extracts and characterized as having indeed RNase activity. The following model was drafted: *S*-glycoproteins are extracellular enzymes that due to a coupled signal sequence can enter the secretory pathway. Thus, immunocytochemical studies revealed that *S*-RNases are secreted into the style mucilage, which separates the files of transmitting tissue cells and provides a matrix through which pollen tubes grow towards the ovary (*Anderson* et al., 1989). The stylar *S*-glycoproteins can penetrate the pollen tube and degrade RNA in the cytoplasm. This would interfere with protein synthesis; however, no *in vivo* evidence had been known to support this hypothesis. Half a year later, the same laboratory was able to present the adequate proof (*McClure* et al., 1990): it was revealed by the use of ^{32}P isotope that ribosomal RNAs from styles of compatible crosses are intact, but degraded in incompatible crosses. Similarly to other rRNA (and not transfer or messenger RNA) targeting strategies for selective toxicity in bacteria and plants, the key action of self-incompatibility also lies in the rRNA degradation. Later functional evidence was also provided by transgenic *Petunia inflata* plants expressing a mutant inactive S_3-RNase, which were consequently unable to reject S_3 pollen. Ribonuclease activity was verified as being the integral part of the *S*-RNase function. A major unresolved question remained the nature of allelic specificity in SI. Authors supported a model in which a ribonuclease mediated cytotoxic effect is responsible for the self-rejection process (referred as the inhibitor model), nevertheless they did not exclude the possibility for a mechanism by which *S*-RNase access to the cytoplasm of pollen tubes is specifically controlled (designated as the gatekeeper model).

As the *S* determinants are glycoproteins with *N*-linked glycan chains containing fucose, arabinose, xylose, mannose, galactose and possibly glucose (*Anderson* et al., 1986), it seemed possible to attribute an important role in the cell–cell recognition to the carbohydrate moiety. It was suggested by the fact that in animals, glycan chains of many glycoproteins are involved in specific recognition between sperm and eggs or in the immune responses (*Wassarman*, 1990; *Ryan & Farmer*, 1991). Immune system of animals is indeed analogous to plants' self-incompatibility with the apparent difference that former rejects non-self, while SI of plants quite the contrary is a rejection of self (*Nasrallah*, 2005). Albeit, enzymic removal of the glycan side chains had no effect on the RNase activity of native *Petunia* *S*-RNases, *Broothaerts* et al. (1991) hypothesized that these chains might be involved in the recognition reaction. The functional evidence that there is an additional pivotal difference between the recognition mechanisms of animal immune system and plant's SI was provided by *Karunanandaa* et al. (1994). By means of genetic transformation they produced *Petunia inflata* plants of the S_1S_2 genotype, in which an accessory engineered S_3 gene was expressed. In this mutant S_3 protein the asparagine codon for the only potential *N*-glycosilation site was replaced with a codon for Asp. Several transgenic lines that produced a normal level of the non-glycosilated S_3 protein acquired the ability to reject S_3 pollen completely. Thus, it was admittedly evidenced that the determinant for the *S*-haplotype specificity resides not in the glycan side chains but in the protein backbone.

Sequence diversity among *S*-RNases is the key element of discrimination between self and non-self. Comparison of 12 solanaceous *S*-RNase sequences identified five conserved domains, named C1 to C5, and two hypervariable regions, termed HVa and HVb (*Ioerger* et al., 1991; *Tsai* et al., 1992). These regions are also the most hydrophilic regions of the *S*-RNase, leading to the hypothesis that HVa and HVb may have a crucial role in determining allelic specificity. *Matton* et al. (1997) could later successfully verify that hypervariable domains indeed mediate allele-specific pollen recognition with experiments using transgenic *Solanum chacoense* plants.

As it was seen, in determining certain details of the SI mechanism incontestable evidences were obtained by the transgene technology. It elucidates why plants belonging to the *Solanaceae* family were used in most experiments rather than species from the *Rosaceae* family. Nevertheless, most findings were validated or seem possible in the *S*-RNase mediated SI system of rosaceous plants.

Broothaerts and his co-workers (1995) published the first paper demonstrating isolation and characterization of *S*-allele cDNA sequences from a species outside the

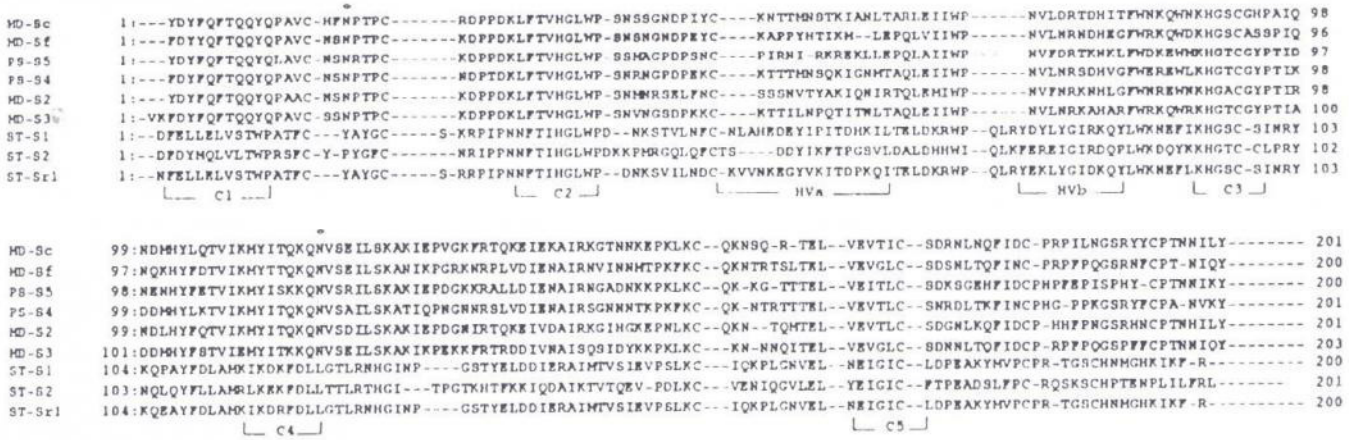

Figure 2 An alignment of the amino acid sequences of *Malus* × *domestica* $S_{c/9}$- and $S_{f/1}$- (MD-Sc and MD-Sf; *Sassa* et al., 1996), *Pyrus serotina* S_5- and S_4- (PS-S5 and PS-S4; Sassa et al., 1996) and *Malus* × *domestica* S_2- and S_3-RNases (MD-S2 and MD-S3; Broothaerts et al., 1995) with *Solanum tuberosum* S_1, S_2 and S_{r1}-RNase (ST-Sr1; Kaufmann et al., 1991). Gaps are marked by dashes. The five conserved regions and the two hypervariable regions reported in the solanaceous *S*-RNases (*Ioerger* et al., 1991) are shown under the sequences of the *S. tuberosum S*-RNases. Two potential *N*-glycosylation sites that are conserved in the rosaceous *S*-RNases are marked with circles over the sequences (Modified after *Sassa* et al., 1996)

Solanaceae family. A conserved region around one histidine residue that is thought to be necessary for RNase activity was identified as being equivalent to C2 of solanaceous *S*-proteins. Regions C2 and C3 of apple *S*-alleles are also present in fungal RNases (*Kawata* et al., 1988) and C3 contains another His residue involved in enzyme catalysis (*Broothaerts* et al., 1995). Based on these two sequence motifs, *S*-RNases were shown to belong to the fungal T_2-RNase gene family, and hence this was named as T_2/*S*-ribonuclease superfamily (*Sassa* et al., 1996). One highly variable domain (HVa) from two of those detected in solanaceous *S*-RNases formed a part of the only hypervariable region of apple S_2- and S_3-RNase sequences (*Broothaerts* et al., 1995).

By cloning and sequencing two *S*-RNases from apple and Japanese pear and aligning their deduced amino acid sequences with available *S*-RNases, striking structural similarities were obtained between the C1 and C5 regions of the solanaceous and those of the rosaceous alleles (*Figure 2*), as well as eight conserved cystein residues and two conserved potential *N*-glycosylation sites (*Sassa* et al., 1996). The *S*-RNases from the *Rosaceae* family showed a degree of sequence diversity ranging from 60.4% to 69.2%, that is consistent with the high degree of allelism resides in the *S* locus.

Further information concerning the *S*-RNase gene structure was acquired when *Ushijima* et al. (1998) cloned and sequenced cDNAs encoding three *S*-RNases of almond (*Prunus dulcis*). An alignment with nine other rosaceous *S*-RNase sequences has revealed that 42 sites were completely conserved among them, from which 25 fell into five distinct regions (*Figure 3*). Positions and sequences of the rosaceous regions C1, C2, C3 and C5 were similar to those of the solanaceous *S*-RNases, but no sequence could be corresponded to the C4 domain of solanaceous *S*-RNases, as it was also shown previously (*Sassa* et al., 1996). However, a conserved region could be identified at a similar position to the solanaceous C4, but this showed no homology to any other T_2/*S*-type RNases or to *S*-like RNases. This *Rosaceae* specific domain was designated as RC4. In this region, there is one potential *N*-glycosylation site conserved among all rosaceous *S*-RNases.

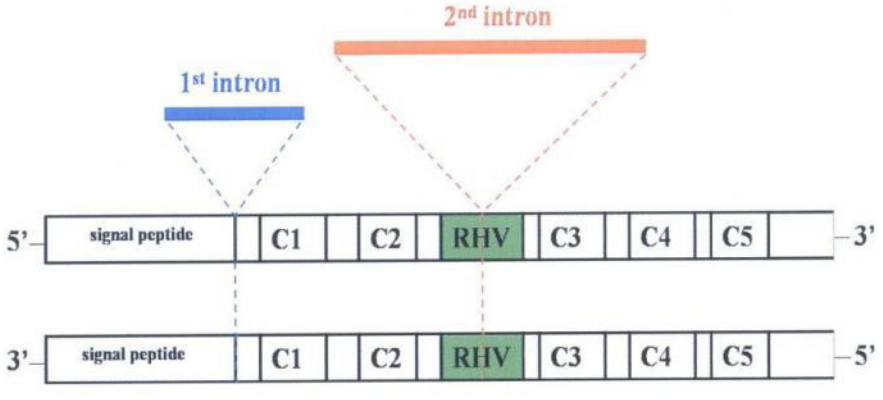

Figure 3 *S*-RNase gene structure of species from the *Prunoideae* subfamily (Modified after *Sonneveld* et al., 2003)

One region involving many variable sites was found in the rosaceous *S*-RNases, named as RHV (*Ushijima* et al., 1998). It corresponds in position to the HVa of solanaceous *S*-alleles, while amino acid sequences in the region corresponding to the solanaceous HVb were invariable in the proteins from *Rosaceae*. As HVa mediates allele-specific pollen recognition in the solanecous species (*Matton* et al., 1997), the corresponding RHV in the rosaceous gene might also play a crucial role in self/non-self pollen discrimination.

Similar cDNA cloning, sequencing or genomic PCR based experiments were carried out in case of several *S*-alleles from more and more rosaceous species, including Japanese pear (*Norioka* et al., 1995; *Sassa & Hirano*, 1997), apple (*i.a. Janssens* et al., 1995; *Verdoodt* et al., 1998; *Matsumoto* et al., 1999; *Matsumoto & Kitahara*, 2000; *Van Nerum* et al., 2001; *Kitahara & Matsumoto*, 2002a,b), almond (*Ushijima* et al., 1998; *Tamura* et al., 2000; *Ma & Oliveira*, 2001; *López* et al., 2004), sweet cherry (*Tao* et al., 1999; *Sonneveld* et al., 2001), sour cherry (*Yamane* et al., 2001; *Hauck* et al., 2002), Japanese apricot (*Yaegaki* et al., 2001) and apricot (*Romero* et al., 2004; *Halász* et al., 2005a,b). The increasing abundance of data confirmed the rosaceous *S*-RNase gene structure being completely identical with that reported by the first studies.

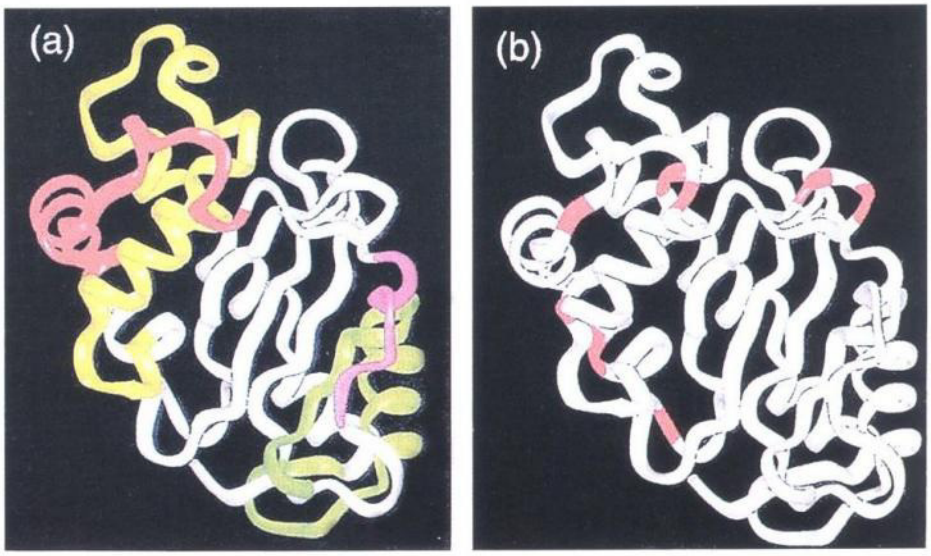

Figure 4 Locations of the four regions under positive selection (PS) within the rosaceous *S*-RNases and the amino acid substitutions between the two highly homologous pairs of *S*-RNases on the tertiary structure of RNase Rh. (a) The PS1, PS2, PS3 and PS4 regions respectively are coloured red, yellow, green and purple. (b) Amino acid substitutions between *Pyrus pyrifolia* S_3- and S_5-RNases are shown in red.

As it was detected in solanaceous *S*-RNases, the C1, C4 and C5 regions of rosaceous *S*-RNases also contain many hydrophobic amino acid residues (nearly 60% of all), suggesting that these residues form the structurally important hydrophobic core of the enzyme and play a pivotal role in stabilizing the enzyme structure (*Ioerger* et al., 1991; *Ushijima* et al., 1998; *Matsuura* et al., 2001). Many hydrophilic amino acids (nearly 60%) are included in the hypervariable region, implying that these are exposed at the surface of the folded protein and play an important role in the discrimination of self from non-self pollen (*Ushijima* et al., 1998). *Figure 4.* shows these regions on the tertiary structure of RNase Rh (*Ishimizu* et al., 1998).

Surprisingly, S_6-RNase and S_{24}-RNase alleles of sweet cherry (*Wünsch & Hormaza*, 2004) as well as S_i- and S_n-RNases from European pear (*Zisovich* et al., 2004) have been found to have an apparently identical hypervariable region (RHV). As it was shown, S_n-RNase does not inhibit the growth of S_i-haplotype pollen, hence presenting a case in which RHV is not required for the determination of specific pollen rejection. *Matsuura* et al. (2001) determined the crystal structure of the S_3-RNase from *Pyrus pyrifolia*, which consists of eight helices and seven β-strands and its folding topology is typical of RNase T_2 family enzymes. Four regions – the hydrophilous PS_1 (RHV), PS_2 and PS_3, as well as the hydrophobic PS_4 – of *S*-RNase could simultaneously interact with multiple pollen factors and the substrate RNA. It is expected that when RHVs are identical, *S*-RNase allele specificity will be determined by one or more of the other PS regions (*Zisovich* et al., 2004). Differences between S_6- and S_{24}-RNase alleles of sweet cherry occur within the 14 amino acids found after the C1 region (*Wünsch & Hormaza*, 2004). This stretch is also highly variable among all *Prunus* *S*-RNases, while in the solanaceous *S*-RNases this region contains two of the most highly variable residues found outside the HVa and HVb hypervariable regions (*Tsai* et al., 1992). Similar pairs of *S*-RNases have also been described in other species of the *Rosaceae*: for example 8 amino acid substitutions in S_3- and S_5-RNases are enough to discriminate between S_3 and S_5 pollens. As it is expected that the highest variable residues must be involved in the allelic specificity, roles for regions outside the RHV are presumable.

The pollen component: an F-box protein

RNA and protein blot analyses with specific cDNAs and antisera have not revealed any pollen component corresponding to *S*-genotype (*Haring* et al., 1990). Expression of *S*-RNases in transgenic plants using their respective promoter causes a change in pistil but not in pollen *S*-specificity (Lee et al., 1994). SC mutants of cherry and Japanese apricot cultivars were characterized to be pollen-part mutants with normal pistil function (*Bošković* et al., 2000; *Ushijima* et al., 2004).

Functional evidence was presented by *Dodds* et al (1999) that in contrast to the previous suppositions the pollen expressed *S*-RNases were not involved in self-incompatibility. They showed that the S_3-RNase of *Lycopersicon peruvianum* is expressed during anther development, then they transformed plants with sense and antisense versions of the S_3-RNase coding region under the control of a pollen-specific promoter. None of the transgenic plants showed an altered self-incompatibility phenotype, demonstrating that S_3-RNase is not the pollen product of the S_3-allele.

A considerable study was published right at the turn of the millennium by *Luu* et al. (2000). By immunocy-

tochemical labelling of pollen tubes growing in styles, they have detected the accumulation of an *S*-RNase in the cytoplasm of all pollen tube haplotypes, thus providing experimental support for the inhibitor model of self-incompatibility. One year later, *Golz* et al. (2001) clarified the essential role of pollen *S*-gene products in the growth of pollen tubes, a fact also being in conflict with the receptor model but compatible with the inhibitor model.

Subsequently, a quite recent focus of attempts to identify the pollen *S*-gene has been to sequence the regions flanking the *S*-RNase gene (*Roalson & McCubbin*, 2003). This approach was based on the prediction that the pollen *S*-gene must be very tightly linked to the *S*-RNase gene, since recombination in this chromosomal region between these two genes would inevitably result in the breakdown of SI (*Kao & Tsukamoto*, 2004). In addition, such recombinant genotypes have never been observed (*de Nettancourt*, 1997). Advances in technology, especially the development of Bacterial Artificial Chromosome (BAC) libraries made chromosome walking more feasible. BAC clones were firstly identified in *Petunia* and *Antirrhinum* plants. Six putative genes were found, from which the most significant was a gene, named *SLF* (*S*-locus F-box), encoding an F-box-containing protein located downstream from the *S*-RNase gene and expressed in pollen and tapetum (*Lai* et al., 2002). F-box proteins are usually components of ubiquitin-ligase complexes, which together with ubiquitin-conjugating enzymes, mediate protein degradation by the 26S proteasome. This sequence motif was revealed in cyclin F, a cell cycle regulator element, and named after this by *Bai* et al. (1996).

Aaron Ciechanover, Irwin Rose and Avram Hershko (native of Hungary) using a reticulocyte lysate system in a series of pioneering biochemical studies discovered and characterized the ATP-dependent, ubiquitin-mediated protein degradation system. The Nobel Prize in Chemistry for 2004 was shared among these three scientists (*Thelander*, 2004).

Eukaryotic cells contain some 6.000 to 30.000 proteins, which are continuously synthesized and degraded and therefore they are in a dynamic state. Protein degradation was known not to be an energy-dependent process. A great astonishment was raised when *Simpson* (1953) found that intracellular protein degradation of cultured liver slices required metabolic energy. Ubiquitin was first isolated from bovine thymus but later found in many tissues of many organisms (*Goldstein* et al., 1975). It is a protein of only 76 amino acids, and its covalent attachment to another protein was first described in 1977 (*Goldknopf & Busch*, 1977).

The multi-step ubiquitin-tagging hypothesis was worked out between 1981 and 1983. Ubiquitin-protein ligation requires the sequential action of three enzymes (*Hershko & Ciechanover*, 1998). The C-terminal glycine residue of ubiquitin is activated in an ATP-requiring step by a specific activating enzyme, E1 (Step 1). The mechanism of the activation itself is very similar to that of precursors within biosynthetic pathways of several biomolecules. This step consists of an intermediate formation of ubiquitin adenylate, with the release of PP_i, followed by the binding of ubiquitin to a Cys residue of E1 in a thiolester linkage, with the release of AMP. Activated ubiquitin is next transferred to an active site Cys residue of an ubiquitin-carrier protein, E2 (Step 2). In the third step catalyzed by an ubiquitin-protein ligase (E3) enzyme, ubiquitin is linked by its C-terminus in an amide isopeptide bound to an ε-amino group of the substrate protein's Lys residues (Step 3).

Usually, there is a single E1, but there are many species of E2s and multiple families of E3s functioning in living cells. These ubiquitin-protein ligases have centrally important roles in determining the selectivity of ubiquitin-mediated proteolysis.

The polyubiquitinated proteins marked to be destroyed are recognized by the 26S proteasomes (*Figure 5*) located both in the nucleus and in the cytoplasm. The approximately 2.5 MDa proteasome consists of several constituents involving the 19S regulatory subcomplexes and the 20S core particle (*Voges* et al., 1999). The previous ones unfold the protein substrates and assist in their translocation through a narrow gate into the 20S core particle where degradation takes place. The active sites of the proteasome are protected from the cellular environment (or conversely, the cellular environment is protected from the active sites of the proteasome) in the interior of a barrel-shaped structure. The proteins are degraded processively until

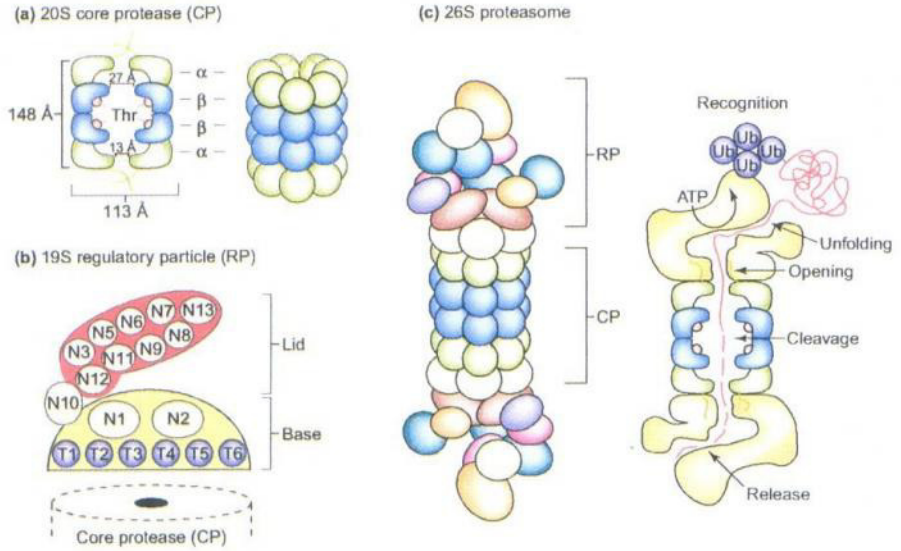

Figure 5 Organization and structure of the 26S proteasome. (a) Organization of the core protease (CP) and its (b) regulatory particles (RP). The N-terminal Thr residues that form the active sites are indicated. N – non ATP-ase RP subunits; T – ATP-ase RP subunits. (c) Proposed structure and events of protein degradation (After *Vierstra*, 2003)

several types of products are generated (Step 4): free peptides, short peptides (e.g. 7–9 amino acid residues) still linked to ubiquitin *via* their Lys residues, and polyubiquitin chains. The latter two products are converted to free and reusable ubiquitin by the action of ubiquitin-C-terminal hydrolases or isopeptidases (Steps 5 and 6). The 19S complex also contains an isopeptidase that removes ubiquitin from the substrate protein. Some isopeptidases may also disassemble certain ubiquitin-protein conjugates (Step 7) and thus prevent their proteolysis by the 26S proteasome. This may have a correction function to salvage incorrectly ubiquitinylated proteins or may have a regulatory role (*Hershko & Ciechanover*, 1998). Short peptides released from the above described processes can be further degraded to free amino acids by cytosolic peptidases (Step 8).

The ubiquitin-protein ligases (E3s in *Figure 6*) have crucial roles in the appropriate functioning of selective protein degradation. Four types are known, from which one is a high-molecular-weight complex involved in the degradation of some cell-cycle regulators, where phosphorylation of the substrate converts it to a form susceptible to the action of the ubiquitin ligase. The so-called SCF E3 enzymes (*Figure 6*) are complexes of four polypeptides that together have ubiquitin-ligase activity. Its name is an abbreviation coming from the name of three of its subunits, *S*KP1, *C*DC53 (or Cullin), and *F*-box protein. The fourth subunit binds E2-ubiquitin. The SCF E3s bring the activated ubiquitin-E2 intermediate together with the targets to promote transfer without forming an E3-ubiquitin intermediate (*Vierstra*, 2003).

As it was mentioned formerly, the F-box protein identifies the target proteins. It binds to the *S*KP1 component of the complex through a degenerate N-terminal motif called the F-box, which contains approximately 60 amino acids (*Gagne* et al., 2002). At its C-terminus it contains several protein-interaction motifs that presumably participate in substrate recognition. The F-box motif binds SKP, thus anchoring the recognition motif to the other two subunits conferring the ubiquitin ligase activity. This provides an effective mechanism for recognizing many protein substrates simply by exchanging F-box subunits (*Vierstra*, 2003).

One may think that the high energy investment in a degradative process is rather strange and a great luxury of the eukaryotic plant cells, considering the well known biochemical thesis stating that all catabolic processes administer energy to the living organisms. But if one also considers that the energy demanding controlled and selective protein degradation removes abnormally folded proteins and involved in various aspects of plant growth, development and defence (*Hare* et al., 2003; *Vierstra* et al., 2003) as well as that the sizable fraction of the plant genome is devoted to encoding these proteins (*Gagne* et al., 2002; *Vierstra*, 2003), from these facts it emerges suddenly that this pathway provides great advantages for the cells far beyond the costs of expended energy.

Lai et al. (2002) were the first, who isolated a gene named *SLF* in *Antirrhinum*. The F-box containing protein was found

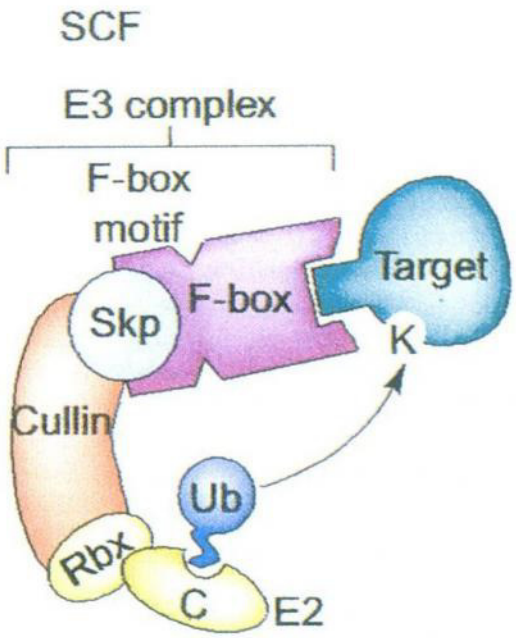

Figure 6 Organization and structure of SCF complex in association with an ubiquitin-E2 intermediate. The pathway of ubiquitin transfer from E2 to the accessible lysines (K) in the target is indicated by the arrow (After *Vierstra*, 2003)

9 kb downstream from the *S*-RNase gene of S_2-haplotype. Despite the low degree of polymorphism, *Ah*SLF and its allelic products have approx. 95% identity at amino acid levels (*Zhou* et al., 2003), the alteration of the pollen function by *Ah*SLF-S_2 clearly showed that this *Antirrhinum* F-box gene is capable of determining male specificity (*Qiao* et al., 2004). F-box proteins were verified to be the pollen component in both of the two other plant families with *S*-RNase mediated SI system, i.e. in the *Rosaceae* (*Entani* et al., 2003; *Ushijima* et al., 2003) and surprisingly at last in the *Solanaceae* (Kao & *Tsukamoto*, 2004; *Sijacic* et al., 2004), which had a path-breaker role in the *S*-RNase studies.

Based on the findings that the *S*-locus is a multigene complex, the term "haplotype" has been adopted to denote variants of the locus, while the term "allele" is used to label variants of a certain polymorphic gene resides at the *S*-locus (*McCubbin & Kao*, 2000). Entani et al. (2003) were the first, who published information on a pollen expressed F-box gene of a plant outside the *Scrophulariaceae* family. They investigated the genomic structure of the *S*-locus region of the S_1- and S_7-haplotypes of Japanese apricot (*Prunus mume*) and identified 13 genes around the *S*-RNase gene. Among them, only one F-box gene (termed *SLF*) fulfilled the requirements for a pollen *S*-determinant gene: it was located within the highly divergent *S*-locus region, it exhibited *S*-haplotype specific amino acid sequence diversity, and it was specifically expressed in pollen but not in styles or leaves. Supposing that SLF functions as the pollen *S*-determinant, the authors concluded that its C terminal region would be expected to contain the recognition site of the target protein, presumably the

S-RNase. A comparison of amino acid sequences suggested that the C-terminal region of SLF contains several highly variable short regions surrounded by conserved sequences, and these highly variable regions are rich in charged amino acids and cysteins, which may interact with the hypervariable regions of *S*-RNases.

Almost at the same time, an F-box gene was identified in almond (*Prunus dulcis*) (*Ushijima* et al., 2003). A 70 kb chromosomal region of cultivar 'Jeffries' ($S_{cm}S_d$) that contains the S_c-RNase is considered the functional region of the *S*-locus (*Ushijima* et al., 2001). This segment was completely sequenced and found to be rich in retro-transposon-like sequences, indicating that almond *S*-locus was subjected to repeated rearrangements, deletions and insertions similarly to *S*-loci of other species. In addition, it contains two pollen-expressed F-box genes that are likely candidates for pollen *S*-genes. One of them was identified as *PdSLF*. This region showed 95.1% identity in case of two different alleles, similarly to 97.9% identity between *Antirrhinum SLF* allelic products (*Lai* et al., 2002). The other F-box motif containing protein was named SFB (*S*-haplotype-specific F-box protein), and located in inverse orientation to the *S*-RNase gene. It contained one intron, and except for the N-terminal F-box motif, showed no significant homology with known proteins. Two regions at the C terminus were variable, as they correspond to the hypervariable region of the *S*-RNase, and hydrophilic enough to be exposed on the molecule's surface. The SFB gene was exclusively expressed in pollen and showed a high level of *S*-haplotype-specific sequence polymorphism, comparable to that of the *S*-RNases.

Yamane et al. (2003) sequenced the flanking region of S_6-RNase from a sweet ('Satonishiki') and a sour ('Rheinische Schattenmorelle') cherry. An F-box motif was found about 380 bp downstream of the gene for S_6-RNase that was not connected to any kind of signal peptides (it is a mutual attribute for all known F-box proteins suggesting their cytoplasmic localization), its direction of transcription was the opposite of that of the S_6-RNase. A single intron of 101 bp was revealed. All these data favoured the assumption that this is an ortholog of *Pd*SFB, and was therefore named as *Pa*SFB$_6$.

To further characterize sweet cherry SFBs, the same research team cloned and sequenced four additional alleles SFB$_1$, SFB$_2$, SFB$_4$, and SFB$_5$ (*Ikeda* et al., 2004). In an amino acid alignment using altogether 13 allele products, 121 out of 384 sites were shown to be conserved and an additional 65 sites had only conservative replacements. Amino acid identity among the SFBs ranged from 66.0% to 82.5%. Only 34 of the non-conserved sites were considered to be highly variable, most of them grouped at the C-terminal region in two variable (V_1 and V_2) and two hypervariable (HVa and HVb) boxes. These variable and hypervariable regions appeared to be hydrophilic or at least not strongly hydrophobic, which suggests that these are exposed on the surface and may have a function in the allele-specific recognition.

The increasing amount of data concerning the nature of the pollen *S*-gene opened the door to precise molecular analysis of self-compatible pollen-part mutant (PPM) cultivars of *Prunus* species. *Ushijima* et al. (2004) analysed two pollen-part mutants: 12 self-compatible cultivars of sweet cherry and 6 of Japanese apricot were studied. Cherry S_4'-haplotype originated from the John Innes Institute (JI), where flower buds of a parental accession ('Napoleon', S_3S_4) were X-ray irradiated (*Lewis*, 1949; *Bošković* et al., 2000). Majority of the PPMs obtained by X-ray irradiation in the *Solanaceae* were associated with competitive interaction that resulted from partial duplication of the *S*-locus (*Golz* et al., 1999, 2001). Genomic DNA blot analysis, however, showed that the cherry S_4'-haplotype did not contain SFB$_3$. Sequence comparison of the mutated and non-mutated SFB$_4$ revealed that four nucleotides were deleted in SFB$_4$', which is located downstream of the S_4-RNase with the opposite direction of transcription as in Japanese apricot, almond and cherry *S*-haplotypes. DNA double-strand breaks induced by ionizing radiation such as X-ray are often repaired via a non-homologous end-joining (NHEJ) pathway in plants, which frequently induces a deletion of short sequence repeats (*Gorbunova & Levy*, 1997). The TTAT deletion is located just before the HVa region and leads to a frame-shift, producing transcripts for a defective SFB lacking the two hypervariable regions and causing a premature termination of the protein due to a stop codon being out of position.

The S_3' is another mutated allele of sweet cherry conferring self-compatibility, in which a rearrangement has been found downstream of the *S*-RNase gene, with the breakpoint of the rearrangement ~3.5 kb from the *S*-RNase gene (*Sonneveld* et al., 2005). A so-called intergenic PCR was conducted to amplify the region between *S*-RNase and SFB genes in the S_3-haplotype in all four possible primer combinations to reveal the relative orientation of the genes and the distance between them. Only the PCR that included the S_3-RNase forward and the S_3-SFB forward primers gave a product of ~8 kb. Consequently, the S_3-SFB gene is located ~6.5 kb downstream of the S_3-RNase gene in opposite transcriptional orientation. Since the same primer set did not give any amplification from the S_3'-haplotype, the S_3-SFB gene of the progenitor was deleted in the mutant offsprings.

Naturally occurring self-compatible Japanese apricot cultivars carry the S_f- allele, which is considered to be undergone a pollen-part mutation. *Ushijima* et al. (2004) completely sequenced a ~9 kbp fragment and found a single gene similar to SFB, designated as SFB$_f$, of which a putative coding region was disrupted in the middle by a ~6.8 kbp insertion. The original F-box sequence was shown to be an SFB, not an SLF. The insertion generated a stop codon, therefore, the SFB$_f$ transcripts encode a putative SFB$_f$ protein that lacks 195 amino acid residues at the C-terminal half and contains 37 additional residues encoded by the inserted sequence. Consequently, the putative SFB$_f$ protein is truncated and lacks the HVa and HVb regions that are supposedly present in the original allele.

Models for the SI reaction and evidences for the bipartite theory

While the discovery of the pistil component of self-incompatibility reactions dates back to 1989; the pollen *S*-gene product has only been recently clarified. *Thompson & Kirch* (1992) in their review dedicated a chapter for the pollen *S*-gene, which was highly theoretical. They stated that genetic analyses of *S*-locus had suggested that it consists of a single specificity domain, driven by a stylar and a pollen activity domain. This could have corresponded to an *S*-RNase structural gene controlled by style- and pollen-specific promoter elements. A drawback to this was that how an *S*-RNase produced within or secreted from the pollen tube could fulfil the specificity determining function of the pollen *S*-gene. The authors noted additionally that no such an RNase protein or transcript had been detected in pollen. Consequently, they concluded that a second coding sequence located in the *S*-locus might determine the pollen component, which either can be a membrane receptor component or an intracellular inactivator of *S*-RNases.

There were proposed two models for gametohytic self-incompatibility, the so-called "receptor" (or gatekeeper) model and the "inhibitor" model (*Thomas & Kirch*, 1992; *Kao & McCubbin*, 1996). The previous one presupposed that the pollen *S*-product is a transmembrane receptor, which allows extracellular *S*-RNase encoded by the same locus to enter the pollen tube. The uptake would result in RNA degradation and growth arrest of pollen tubes. This model seemed less probable as in case of diploid pollens, the pollen tubes carrying receptors for two *S*-RNases proved to be compatible. The inhibitor model predicts that the pollen *S*-product is a general *S*-RNase inhibitor that resides within the growing pollen tube. The model proposes that all *S*-RNases can freely enter the pollen tube, and pollen *S*-product binds and inhibits all *S*-RNases except for those encoded by the same or genetically identical locus. Therefore, these will degrade RNA and inhibit pollen tube growth.

Broothaerts et al. (1991) still have presumed that the allelic specificity resided on *S*-RNase uptake by the pollen tubes. *Lewis* (1960) envisaged the gametophytic *S*-locus as having three parts, a part expressed in the pistil, another expressed in the pollen and a third one encoding the allelic specificity. This tripartite model was based on the findings that pollen and style SI functions could be mutated independently of each other. The product of the pollen and the stylar parts of the *S*-locus might be identical and in incompatible mating they form an active dimer. Nevertheless, several evidences enumerated thereinafter indicate that the pollen and the pistil components are separate genes.

The modified inhibitor model was drawn by *Luu* et al. (2001), who used transgenic *Solanum* plants expressing chimerical *S*-RNases with dual-specificity. This model predicts that the active form of pollen *S*-allele product is a homotetramer, which carries the *S*-allele-specificity domain, and a general inhibitor is responsible for the inhibition of *S*-RNases. This general inhibitor would bind and inhibit all *S*-RNases unless an *S*-RNase was bound to its cognate pollen *S*-allele product through their matching domains. When diploid pollens resulting from tetraploid plants express two different alleles of the pollen *S*-gene, the products would mainly form heterotetramers, which could not efficiently bind either cognate *S*-RNase and the general inhibitor would inhibit both of cognate *S*-RNases. Thus, pollen tubes carrying two different *S*-alleles would be compatible with pistils of any *S*-genotype. The dual-specificity *S*-RNase could bind the heterotetramers formed by the two pollen *S*-allele products, binding of the general inhibitors to the *S*-RNase would be blocked, explaining the SI behaviour (*Kao & Tsukamoto*, 2004).

Complementing the previously reviewed evidences, F-box descriptive experiments provided compelling proofs for the inhibitor model of self/non-self recognition under gametophytic self-incompatibility. It assumes that non-self *S*-RNases are polyubiquitinated by SCF_{SFB} to be degraded by the 26S proteasome pathway, which results in inactivation of the non-self *S*-RNases (*Ushijima* et al., 2004). Concurrently, the activity of the cognate *S*-RNases is unaffected by the *S*-haplotype-specific interaction in the pollen tube. This model is similar to the inhibitor model in that the compatible interaction provokes the inactivation of *S*-RNases, while the incompatible reaction does not. Since the SFB_f from Japanese apricot and SFB_4' from sweet cherry encode a truncated SFB, an SCF complex containing SFB that lacks HVa and HVb regions could lose the haplotype-specific interaction domain, but retain the general interaction domain, consequently, polyubiquitinate not only non-self *S*-RNase but also cognate *S*-RNase for subsequent degradation by the 26 S proteasome pathway (*Figure 5C*). An alternative scenario is also possible: it is known that misfolded, defective proteins such as SFB_f and SFB_4' are readily degraded in the cells. In this case SFB could act as a blocker to prevent degradation of cognate *S*-RNase by a so-called general inhibitor, an unknown molecule that inactivates all *S*-RNases (*Ushijima* et al., 2004).

Qiao et al. (2004) provided evidence that ubiquitin/26S proteasome activity is essential in compatible but not in incompatible interactions in *Antirrhinum*. In contrast to the previously described model drawn by *Ushijima* et al. (2004), *Sonneveld* et al. (2005) presuppose that SFB is not the inactivator of non-self *S*-RNases and indicate the presence of a general inactivation mechanism, with SFB conferring specificity by protecting self *S*-RNases from inactivation. This hypothesis is supported by the argument that in the original inhibitor model a loss of function of the haplotype-specific F-box gene should lead to universally incompatible pollen instead of a universally compatible one, because pollen tubes would lack a mechanism to inhibit *S*-RNases. This new model suggests a role for SFB proteins to prevent self *S*-RNases from being degraded, and not recruit non-self *S*-RNases for degradation as it is dictated by other models. However, this has also several unclear details and further

biochemical investigations are required to shed light on the mechanism of *S*-RNase inactivation and the precise role of SFB in protecting self *S*-RNases from degradation.

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