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REVIEW PAPER

Molecular bases and evolutionary dynamics of self-incompatibility in the Pyrinae (Rosaceae)

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Abstract

The molecular bases of the gametophytic self-incompatibility (GSI) system of species of the subtribe Pyrinae (Rosaceae), such as apple and pear, have been widely studied in the last two decades. The characterization of S-locus genes and of the mechanisms underlying pollen acceptance or rejection have been topics of major interest. Besides the single pistil-side S determinant, the S-RNase, multiple related S-locus F-box genes seem to be involved in the determination of pollen S specificity. Here, we collect and review the state of the art of GSI in the Pyrinae. We emphasize recent genomic data that have contributed to unveiling the S-locus structure of the Pyrinae, and discuss their consistency with the models of self-recognition that have been proposed for *Prunus* and the Solanaceae. Experimental data suggest that the mechanism controlling pollen–pistil recognition specificity of the Pyrinae might fit well with the collaborative 'non-self' recognition system proposed for *Petunia* (Solanaceae), whereas it presents relevant differences with the mechanism exhibited by the species of the closely related genus *Prunus*, which uses a single evolutionarily divergent F-box gene as the pollen S determinant. The possible involvement of multiple pollen S genes in the GSI system of Pyrinae, still awaiting experimental confirmation, opens up new perspectives to our understanding of the evolution of S haplotypes, and of the evolution of S-RNase-based GSI within the Rosaceae family. Whereas S-locus genes encode the players determining self-recognition, pollen rejection in the Pyrinae seems to involve a complex cascade of downstream cellular events with significant similarities to programmed cell death.

Key words: F-box, pollen–pistil interaction, Pyrinae, Rosaceae, self-incompatibility, SFBB, S-locus, S-RNase.

Introduction

The Pyrinae subtribe forms a wide and diverse lineage of the Rosaceae family (Potter *et al.* 2007), including ~1000 species belonging to 30 different genera, with a pome-type fruit and a base chromosome number of $x=17$ as the main distinctive characteristics. These include some economically important tree and shrub species, widely cultivated as fruit crops or appreciated as ornamentals, such as apple (*Malus*×*domestica* Borkh.), European and Asian pears (*Pyrus communis* L., *P. pyrifolia* Nakai, *P.*×*bretschneideri* Rehd.), quince (*Cydonia oblonga* Mill.), loquat (*Eriobotrya japonica* Lindl.), and medlar (*Mespilus germanica* L.). The species of Pyrinae exhibit a self-incompatibility (SI) system that prevents self-fertilization through a pollen–pistil recognition mechanism, resulting in the selective inhibition of the growth of those pollen tubes that are recognized by the pistil as 'self' (De Nettancourt, 2001). The prevalence and diversity of SI mechanisms in plants suggest that the evolutionary advantage coming from obligate outcrossing offsets the short-term advantage of selfing (Goldberg *et al.*, 2010). Thus, it has been estimated that genetically controlled SI systems have evolved at least 21 times independently in angiosperms (Weller *et al.*, 1995), highlighting the importance of allogamy in the evolution of flowering plants. Nevertheless, from an agronomic point of

view, SI can be an undesired trait for those cultivated species, like those of the Pyrinae, for which fruit set and fruit development are highly dependent on seed set, and hence on a successful fertilization process. Because of its interest both for academia and agriculture, SI has been intensively studied in a wide range of species including some relevant crop plants.

Like other groups of the Rosaceae, the members of the Pyrinae exhibit the so-called S-RNase-based gametophytic self-incompatibility (GSI), which is considered to be the most widespread SI system among flowering plants. Moreover, S-RNase-based GSI has also been characterized so far in two other families distantly related to the Rosaceae, the Solanaceae and the Plantaginaceae (De Nettancourt, 2001), suggesting an early origin of this SI mechanism in the evolutionary history of angiosperms (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002; Vieira *et al.*, 2007). In GSI systems the specificity of pollen–pistil recognition depends on the interaction between male (pollen-expressed) and female (pistil-expressed) determinants, whose coding genes typically lay in a single and multiallelic locus (S-locus); pollen tube rejection occurs whenever there is a match between the S specificity expressed by the haploid genome of the pollen and one of the S specificities expressed by the diploid genome of the pistil tissue. Much of our knowledge on S-RNase-based GSI came from studies in genera of the Solanaceae (such as *Petunia* and *Nicotiana*) and Plantaginaceae (*Antirrhinum*), which present some advantages as model systems for the study of genetics and molecular biology, namely a short life cycle and the possibility of carrying out large-scale mutagenesis and transformation experiments. In the Rosaceae, the practical interest in SI for breeders and growers has traditionally stimulated research on this topic, although the woody habit of the *Prunus* and Pyrinae species has been a limitation in developing genetic experiments. Interestingly, the existence of a number of induced and spontaneous self-compatible mutants mainly in species of the genus *Prunus* has provided a valuable plant material for the characterization of the SI genes at the molecular level, being particularly useful in the identification of the *Prunus* pollen S determinant. In contrast, the repertoire of self-compatible mutants in the Pyrinae seems to be far smaller, particularly for those mutants affecting pollen function. Thus far, loss-of-function pollen-part mutants have not yet been characterized at the molecular level in this group; this, together with an extremely complex S-locus structure, is limiting research on the pollen S determinant, and as a consequence our knowledge of the molecular basis of Pyrinae SI is at present several steps behind that of other taxa. In the last few years, however, significant advances have been made towards understanding the SI system of this subtribe; the increasing amount of genetic information available on the S-locus genes of *Malus* and *Pyrus*, together with the recent findings reported in model species of the Solanaceae, promise additional advancements in the near future.

Here, we collect and review the state of the art of GSI in the Pyrinae. We emphasize recent genomic data that have contributed to unveiling the S-locus structure of the Pyrinae, and discuss their consistency with the models of self-recognition that have been proposed for *Prunus* and the Solanaceae. We stress the specific characteristics of the SI system in the Pyrinae in relation to these other groups, emphasizing the contributions that studies in this subtribe have made to our general understanding of S-RNase-based GSI. The currently available information, moreover, makes it possible to discuss the features of the S-locus structure of Pyrinae, in the context of recent models involving multiple pollen S genes in SI.

The Pyrinae S-locus genes

Seminal genetic studies in different species with GSI characterized the complexity of the S-locus structure some time ago. Mutagenesis studies showed that the S locus can hold self-compatible mutations impaired independently in pistil and pollen functions, which highlighted that different genes encode the two functions (Lewis, 1960, Pandey, 1962, and references therein). These early studies also predicted that the pistil and the pollen determinants should interact in an S-haplotype-specific manner in order to elicit the SI recognition reaction. In the last 25 years, the molecular characterization of the S-locus region in species of the Solanaceae, Plantaginaceae, and Rosaceae has identified the pistil S determinant of these families as a stylar-expressed RNase, called S-RNase (Anderson *et al.*, 1986; Sassa *et al.*, 1992; Ushijima *et al.*, 1998), and the pollen S determinant as one or multiple pollen-expressed S-locus F-box genes called SLF (for S-locus F-box) in the Solanaceae and Plantaginaceae (Lai *et al.*, 2002; Sijacic *et al.*, 2004), SFB (for S-haplotype-specific F-box) in *Prunus* (Ushijima *et al.*, 2003), and SFBB (for S-locus F-box brothers) in the Pyrinae (Sassa *et al.*, 2007; see Fig. 1).

The S-RNase

The pistil S determinant of S-RNase-based GSI was first identified in *Nicotiana alata* as a stylar glycoprotein of ~30 kDa, showing allele-specific polymorphic patterns associated with the S locus (Bredemeijer and Blaas, 1981; Anderson *et al.*, 1986); unexpectedly its protein sequence was found to exhibit homology with the later characterized sequence of the RNase-T2 of *Aspergillus oryzae* (McClure

Fig. 1. Structure of the S-locus of Pyrinae. The diagram refers to the 378 kb region surrounding the S_2-RNase of Japanese pear, as determined by Okada *et al*. (2011). Each S haplotype harbours a single pistil S gene (the S-RNase, in orange) and a pool of F-box genes (SFBBs, in dark grey). It is noteworthy that the number, order, and orientation of SFBB genes relative to the S-RNase seem to be highly variable between haplotypes.

et al., 1989). *In vitro* testing confirmed that the native protein exhibits RNase activity (McClure *et al.*, 1989) and therefore it was renamed S-RNase, giving a trademark for the S-RNase-based GSI. Transformation experiments in *Nicotiana* and *Petunia* showed that silencing the S-RNase gene leads to the breakdown of SI due to the inability of the pistil to reject self-pollen, whereas expression of a new S-RNase allele in pistils confers a new specificity of pollen rejection (Lee *et al.*, 1994; Murfett *et al.*, 1994); together all these pieces of evidence demonstrated that the S-RNase is the female determinant of GSI in Solanaceae and that its activity is sufficient for determining the pistil S-specificity.

In the early 1990s, Sassa and co-workers found polymorphic stylar RNases in Japanese pear, a member of the Pyrinae, associated with the S alleles of cultivars, leading to the identification for the first time of an SI-associated S-RNase in a species outside the Solanaceae (Sassa *et al.*, 1992, 1993). This result also first highlighted that two distantly related families, the Solanaceae belonging to the Asterid clade and the Rosaceae belonging to the Rosid clade, could actually exhibit similar molecular mechanisms in their GSI systems. The subsequent identification of the S-RNase gene in apple (Broothaerts *et al.*, 1995), Japanease pear (Sassa *et al.*, 1996), and European pear (Zuccherelli *et al.*, 2002) by cloning cDNA and genomic sequences facilitated the evaluation of sequence polymorphisms at the S locus, allowing demonstration of the association between the S-RNase genotypes of cultivars with their cross-(in)-compatibility behaviour. Further evidence confirming that the S-RNase is indeed the pistil S determinant of SI in the Pyrinae came from the functional analysis of different pistil-part mutations in pears and transgenic plants in apple. Thus, the cultivar 'Osa Nijisseiki' of Japanese pear that lacks the S-RNase in the S_4^{sm} haplotype is self-compatible due to the inability to reject the S_4 pollen (Sassa *et al.* 1997). Similarly, in the European pear, lack of expression of the S-RNase in a spontaneous S-allele mutation is related to the breakdown of the pistil function but not the pollen function (Sanzol, 2009*a*). Moreover, the complete silencing of pistil S-RNase expression in transgenic apple trees results in self-fertility (Broothaerts *et al.*, 2004*a*).

The S-RNase is specifically expressed in the transmitting tissue of the style and the protein is post-translationally modified through glycosylation in one or more residues (Ishimizu *et al.*, 1999*b*); even though glycosylation seems not to be essential for its function (Karunanandaa *et al.*, 1994), it may influence pollen rejection thresholds (Liu *et al.*, 2008). The mature protein is secreted in the extracellular matrix, where it comes into contact with growing pollen tubes, to which it is imported in a non S-allele-specific manner (Luu *et al.*, 2000; Goldraij *et al.*, 2006). Like other members of the T2 family of RNases (Sassa *et al.*, 1996), the S-RNase maintains the typical tertiary structure made up of eight α-helices and seven β-sheets, as determined for the S_{F11} allele of *N. alata* (Ida *et al.* 2001) and the S_3 allele of *P. pyrifolia* (Matsuura *et al.*, 2001). Point mutations in the catalytic site result in the inability to trigger pollen rejection, indicating that the RNase activity is required for the pistil S function (Huang *et al.* 1994); on the basis of this finding it was hypothesized that S-RNases may act as specific cytotoxins in incompatible pollen tubes, degrading the cellular RNA. However, since the active site of the S_3-RNase of *P. pyrifolia* was found to exhibit a very similar overall shape to those of other proteins of the RNase-T2 family, it was suggested that S-allele specificity is not elicited at the level of substrate recognition (Matsuura *et al.*, 2001). Moreover, it is also uncertain whether RNA degradation is the sole event triggering pollen tube growth inhibition or if it is just part of a more complex cascade of events that follow an upstream mechanism of specific (non-)self-pollen recognition (see later).

The S-RNase gene shows an extremely high degree of allelic sequence variability, which seems to be the result of long periods of evolution under frequency-dependent balancing selection, and is probably a key aspect in determining allele recognition specificity (Ioerger *et al.*, 1990). The sequence alignment of multiple alleles of Rosaceous S-RNases provide evidence for a protein secondary structure with five consensus conserved regions (C1, C2, C3, RC4, and C5) and one hypervariable region (RHV). Four of the five conserved regions (all except RC4) are shared with the S-RNases of Solanaceae and Plantaginaceae. It is worth noting that the S-RNase of Pyrinae also exhibits an additional highly conserved non-canonical hexapeptide (IIWPNV) region located immediately downstream of RHV, which has been extensively exploited for the development of consensus primers for PCR-based S-genotyping procedures (Ishimizu *et al.*, 1999*a*). In contrast to the S-RNases of Solanaceae and Plantaginaceae that have two hypervariable regions (HVa and HVb), the Pyrinae S-RNase only has one; however, additional highly variable sites in the Pyrinae S-RNase are known to be located throughout the whole protein sequence (Ishimizu *et al.*, 1998; Vieira *et al.*, 2007, 2010). Interestingly, although the canonical RHV/HV regions are known to play a role in determination of S specificity (Ioerger *et al.*, 1991; Matton *et al.*, 1997), both in European pear and apple two pairs of functionally distinct S-RNase alleles were found to share identical RHV regions, highlighting that variable residues in other regions of the protein are involved in the same way in recognition (Zisovich *et al.*, 2004*a*; Matsumoto *et al.*, 2010). According to a putative role for the hypervariable sites in recognition, they are often exposed on the protein surface. Therefore, it is likely that they are involved in the selective interaction of the S-RNase with the pollen S protein (Ishimizu *et al.*, 1998), in agreement with models that predict S haplotype self-recognition through the interaction of the pollen S and the pistil S determinants (Kao and Huang, 1994).

The coding region of the Pyrinae S-RNase is interrupted by a single intron highly polymorphic in length, which is located within the RHV region. The high level of sequence diversity and variation in intron length have been used extensively in evaluating the S-locus diversity in species of the subtribe. Thus, molecular assays for S-genotyping have been developed in species such as apple (Janssens *et al.*,

1995; Verdoodt *et al.*, 1998; Matsumoto *et al.*, 1999*a*, *b*, 2003*a*, *b*; Kitahara *et al.*, 2000; Broothaerts, 2003; Broothaerts *et al.*, 2004*b*; Kim *et al.*, 2006, 2009; Long *et al.*, 2009), European pear (Zuccherelli *et al.*, 2002; Zisovich *et al.*, 2004*b*; Sanzol *et al.*, 2006; Takasaki *et al.*, 2006; Moriya *et al.*, 2007; Mota *et al.*, 2007; Sanzol and Robbins, 2008; Goldway *et al.*, 2009; Sanzol, 2009*a*, *b*, 2010), Japanese pear (Ishimizu *et al.*, 1999*a*; Castillo *et al.*, 2001; Kim *et al.*, 2002, 2007; Takasaki *et al.*, 2004; Gu *et al.*, 2009), loquat (Carrera *et al.*, 2009; Gisbert *et al.*, 2009), or wild populations of *Sorbus* and *Crathaegus* (Raspé and Kohn, 2002, 2007), facilitating the analysis of the S-locus diversity in the subtribe.

Given that the S-RNase is encoded by a single copy gene, it provides a convenient tool for monitoring the allelic diversity and the evolutionary history of the S locus. In natural populations, the S locus is subject to frequency-dependent balancing selection. This means that pollen bearing a rare specificity has increased mating chances, because only a small proportion of the plants will reject it; thus, the frequency of this specificity will increase over generations (Wright, 1939). Conversely, a frequent S specificity will tend gradually to reduce its prevalence in the population. This type of selection results in the maintenance of a high number of S haplotypes in populations for extremely long periods of time. The analysis of S-RNase alleles of Pyrinae species clearly showed the imprint left by frequency-dependent balancing selection on the S-RNase gene; first, it proved to be highly polymorphic: allelic polymorphism at the S locus is maintained because the increase in new S specificities is strongly favoured by balancing selection. Moreover, several sites in the S-RNase gene (of note those in the RHV and other highly variable residues) show high non-synonymous to synonymous nucleotide substitution rates (K_a/K_s), as expected for genes under positive selection (Vieira *et al.*, 2007, 2010); amino acid replacement is expected to be favoured in S gene-encoded proteins, because it would allow the generation of new specificities that would have the chance to spread rapidly in the population. Also, the phylogeny of S-RNase alleles of different Pyrinae species provided evidence of shared ancestral polymorphisms (Sassa *et al.*, 1996; Raspé and Kohn, 2002; Bokszczanin *et al.*, 2009; De Franceschi *et al.*, 2011*a*), resulting in a tree structure showing a pattern of transgeneric/specific evolution (i.e. an allele from one species is often more closely related to alleles from other species or genera than to other alleles of the same species), which suggests S-locus diversification in the subtribe prior to the time of genera divergence (Ishimizu *et al.*, 1998; Raspé and Kohn, 2002). Therefore, it is likely that much of the molecular variability associated with extant S-RNase alleles of the Pyrinae species originated in a common ancestor. At present, the time of diversification of the S locus of Pyrinae is uncertain; nevertheless, since *Prunus* and Pyrinae S-RNases form monophyletic clusters, it should have occurred some time after the split of these two Rosaceous groups (Igic and Kohn, 2001).

The S-locus F-box genes

Whereas the characterization of the S-RNase as the pistil S determinant originally used a protein-based approach, the characterization of candidate genes for the pollen S (i.e. the male determinant of S specificity) has mainly involved the use of map-based approaches that have scanned the genomic region surrounding the S-RNase gene. At the beginning of the last decade, several groups working with different systems found that the S-locus genomic region of species of the Solanaceae (Wang *et al.*, 2004), the Plantaginaceae (Lai *et al.*, 2002), and the Rosaceae (Entani *et al.*, 2003; Ushijima *et al.*, 2003) contains pollen-expressed genes belonging to a family of proteins with an F-box domain. Initially, different lines of evidence suggested that the F-box gene located immediately downstream of the S-RNase was the pollen S in *Petunia*, *Antirrhinum*, and *Prunus*; these genes were named SLF in *Petunia* and *Antirrhinum* for S-locus F-box, and SFB in *Prunus* for S-haplotype-specific F-box. In *Prunus*, the analysis of pollen-part self-compatible mutants showed that breakdown of the pollen activity is associated with loss-of-function mutated SFB alleles (Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005). On the other hand, in *Petunia* and *Antirrhinum*, transgenic experiments showed that SLF elicits competitive interaction when an SLF variant is expressed in pollen harbouring a different specificity, which was the expected behaviour for the pollen S of these species (Sijacic *et al.*, 2004; Qiao *et al.*, 2004; see below). Interestingly, F-box proteins are known to be a component of the E3 ligase complex, named Skp1–Cullin–F-box (SCF), which is involved in protein degradation through the ubiquitin–26S proteasome-dependent pathway. Based on this finding, SLF/SFB were suggested to be a component of an SCF complex (Huang *et al.*, 2006), which led to the proposal of a model for S-haplotype self-recognition involving selective protein ubiquitination and degradation of non-self S-RNases (reviewed in Hua *et al.*, 2008).

In the Pyrinae, the characterization of F-box genes within the S locus was first reported by Cheng *et al.* (2006); using a PCR-based approach, these authors cloned two pollen-expressed F-box genes linked to the apple S_1 and S_2 haplotypes (SLF1 and SLF2). However, Sassa *et al.* (2007) were the first to report a physical characterization of the S-locus structure in this group, and to provide evidence of multiple and related F-box genes within the S locus of apple and Japanese pear. In a bacterial artificial chromosome (BAC) library of the apple cultivar 'Florina' they identified two F-box genes linked to each of the S-RNases S_3 and S_9, and named them MdSFBB (*Malus*×*domestica* S-locus F-box brothers) -α and -β. Moreover, three homologous but distinct genes were identified in Japanese pear, linked to the S_4 and S_5 haplotypes, in this case via a PCR-based approach (PpSFBB-α, -β, and -γ, 'Pp' standing for *Pyrus pyrifolia*). SFBBs were considered good candidates for the pollen S since they exhibited linkage to the S-RNase, pollen-specific expression, and S-haplotype-specific polymorphism. Lack of functional evaluation of the multiple

candidate SFBBs raised the question of whether only one or several of these genes may actually be responsible for the pollen S function of this group. Nevertheless, these sequences allowed the development of S-genotyping molecular assays based on F-box genes rather than on the S-RNase in Japanese pear (Kakui *et al.*, 2007), European pear (Zisovich *et al.*, 2009), and apple (Li *et al.*, 2010).

Two recent papers have provided a detailed analysis of the gene composition of large genomic regions of S haplotypes of apple (Minamikawa *et al.*, 2010) and Japanese pear (Okada *et al.*, 2011). Both studies have agreed in characterizing a large number of SFBB-related genes surrounding the S-RNase in the two species (Fig. 1). Thus, up to 12 and 10 F-box genes were found associated with haplotypes of apple and Japanese pear, respectively. In apple, Minamikawa *et al.* (2010) analysed in more detail the 'Florina' genomic library initially used by Sassa *et al.* (2007), and discovered 20 additional F-box genes, tentatively named FBX1–FBX20, linked to the S-RNase: 10 of them belonged to the S_3 and nine to the S_9 haplotype, the last one (FBX11) being present in both haplotypes. Even though the BAC clones containing these genes could not be assembled into unique contigs associated with each of the two S haplotypes, the linkage of FBXs with the S-RNase and their physical position within or near the S locus were determined through segregation analyses and FISH (fluorescent *in situ* hybridization). Some pairs of homologous genes could be identified between those belonging to the two haplotypes, each putatively derived from a common ancestor, suggesting that proliferation of F-box genes has pre-dated S-haplotype diversification. Nevertheless, their order and position relative to the S-RNase were, at least in some cases, different; thus, the authors concluded that extensive rearrangements may have occurred during evolution, modifying both the order and repertoire of F-box genes within each haplotype. Importantly, all the FBX genes were found to be specifically expressed in pollen, with the only exception of FBX4, which encodes a truncated protein because of in-frame stop codons and can thus be considered a pseudogene.

The parallel study by Okada *et al.* (2011) analysing the structure of the S locus of Japanese pear yielded similar results. These authors fully sequenced two BAC contigs of 649 kb and 378 kb surrounding the PpS_4- and PpS_2-RNases respectively. In the 649 kb region surrounding the S_4-RNase they found, besides the previously described S_4-Fbox0 (Okada *et al.*, 2008), five more SFBB-related genes; in the S_2-haplotype contig, despite its smaller size (378 kb), 10 F-box genes could be identified, five placed upstream and five downstream of the S_2-RNase (Fig. 1). Even in this case, pairs of homologue genes could be identified between the two haplotypes, but no co-linearity was found between the two contigs. The presence of 40 and 20 transposon-like sequences within the S_4 and S_2 contigs, respectively, led the authors to hypothesize that transposable elements might have played a role in haplotype diversification, and might contribute to suppression of recombination at the S locus. Interestingly, the evolutionary pattern showed by SFBBs both in apple and in Japanese pear confirmed the initial findings of Sassa *et al.* (2007) for apple, with some SFBBs within S haplotypes being more closely related than other SFBBs belonging to different S haplotypes. Moreover, different S haplotypes often bear SFBBs showing sequence identities much lower than the identities exhibited by their corresponding S-RNases (De Franceschi *et al.*, 2011*a*).

It is unlikely that all the SFBBs found in the Pyrinae S locus are involved in SI even though they were initially considered as good candidates for the pollen S. A recent analysis of sequence diversity and genetic linkage of multiple SFBBs in different species of the Pyrinae (De Franceschi *et al.*, 2011*a*, *b*) found evidence that some of them are not consistent with a role in SI. Up to six SFBBs linked to a single S haplotype of European pear were characterized, homologous to both the apple and the Japanese pear SFBBs; however, homologues of PpSFBB-α and -γ were shown to be tightly, but not fully linked to the S-RNase gene, suggesting that they are located in close proximity, but outside the S locus. Moreover, their phylogenetic profiles were markedly different from the trans-specific clustering exhibited by the S-RNase and other SFBBs that are located within the S locus, highlighting a clear separation between the *Malus* and the *Pyrus* sequences, and the overall sequence polymorphism was much lower than expected for an S gene.

Based on these findings, each S haplotype seems to exhibit a repertoire of polymorphic SFBBs, some of them positioned at different locations relative to the S-RNase. Given that SFBBs seem to be much younger than the S-RNase (De Franceschi *et al.*, 2011*a*), it is uncertain whether genomic rearrangement has altered the ordering of SFBBs or, alternatively, independent duplication events inserted SFBBs at different positions in different S haplotypes during the diversification of the S locus. Also, the repertoire of SFBBs and the total number seem not to be strictly identical, although genomic sequencing of complete S haplotypes will be needed to evaluate this last possibility in more detail. It is interesting that a number of SFBBs are present, in at least one copy, in the majority of S haplotypes of different species (apple, and Japanese, European, and Chinese pear). This suggests that proliferation of SFBB genes pre-dated speciation in the Pyrinae (De Franceschi *et al.*, 2011*a*), consistent with the trans-specific evolution of the S locus in the subtribe. It is reasonable to hypothesize that a first pool of S-locus F-box genes was established in a common ancestor; subsequent S-haplotype diversification may have involved extensive rearrangements that shuffled some SFBBs within S haplotypes. Alternatively, a dynamic process of gene duplication across S haplotypes during diversification may have placed SFBB genes at different locations relative to the S-RNase; this last scenario would explain why S haplotypes with highly divergent S-RNases actually exhibit much more closely related SFBBs.

The biological meaning of this great number of F-box genes in the Pyrinae S locus initially was unclear, given that at that time a single SLF/SFB had been proposed as the pollen S in *Petunia*, *Antirrhinum*, and *Prunus* (Sassa *et al.*

2007); moreover, none of the SFBB genes fulfilled the expected molecular and genetic features of the pollen S (see later in this review). However, recent findings in *Petunia* have provided convincing evidence that the self-(in)compatible recognition reaction of this species may involve multiple F-box genes, rather than a single one, acting together as the pollen S (Kubo *et al.*, 2010; see below). On the basis of these results, these authors proposed a new model by which each F-box gene within an S haplotype would interact with a subset of non-self S-RNases eliciting a detoxifying effect. It is tempting to speculate about the possibility of extending these findings to the Pyrinae as well, allowing a re-interpretation of the SI system of this group according to the data on its S-locus structure, in which multiple SFBBs co-exist and could be involved in jointly determining the pollen S function.

The molecular pollen–pistil recognition system

Unravelling the molecular basis of the interaction between the female and male S determinants and the mechanism by which this interaction triggers the acceptance or rejection of the pollen tube are important issues yet to be elucidated. A key finding in the development of extant molecular models for S-RNase-based GSI was the observation that S-RNases are transported from the transmitting tissue of the style into the pollen tube in a non-S-allele-specific manner (Luu *et al.*, 2000). This result showed that the recognition between the S-RNase and the pollen S determinant(s) should occur inside the pollen tube, where only self S-RNases would be able to elicit cytotoxic activity. This observation was relevant as it led to support for the existence of some kind of inhibitory mechanism that would impair the activity of non-self S-RNases, but not the activity of self S-RNases. Most important in supporting this hypothesis was the characterization of the pollen S as an F-box gene, which represented a suitable piece to fit into the puzzle: F-box proteins are best known for their participation in protein degradation through the ubiquitin–26S proteasome pathway (Zhang *et al.*, 2009). It thus became feasible that allele-specific recognition of the S-RNase by the F-box component could mediate its ubiquitination inside the pollen tube, marking it for subsequent degradation. Thus, the acceptance of a pollen tube might depend on its ability to degrade the non-self S-RNases produced by the pistil. At present, a widely accepted hypothesis is that protein degradation plays a key role in regulating allele-specific S-RNase activity (reviewed by Zhang *et al.*, 2009).

Several biochemical models have been proposed which attempt to put together the molecular data with the phenotypic behaviour of S-RNase-based GSI (see Hua *et al.*, 2008 for a comprehensive review). These models initially took into account the interaction between the S-RNase and a single F-box protein as the male determinant: this had been considered the most likely hypothesis until recently, given that *Prunus* self-compatible pollen-part mutants were found to be affected in a single F-box gene (SFB), and initial experiments in *Petunia* and *Antirrhinum* demonstrated that SLF fulfilled the expectations for the pollen S of these species. Thus, the function of SLF was tested empirically by genetic transformation exploiting the well-documented phenomenon of competitive interaction that results in breakdown of SI in tetraploid plants: these produce diploid pollen grains, some of them carrying two different haplotypes at the S locus (heteroallelic pollen); the presence of two different S specificities within a pollen tube allows it to be accepted even by 'self' pistils, while two copies of the same S haplotype (homoallelic pollen) do not affect the normal SI phenotype. AhSLF-S2 from *Antirrhinum hispanicum* and PiSLF2 from *Petunia inflata* were found to be able to trigger competitive interaction when coupled with different S haplotypes (Qiao *et al.*, 2004; Sijacic *et al.*, 2004); thus, since competitive interaction is known to occur due to the expression of two distinct pollen S specificities within the same pollen tube, the two genes from *Petunia* and *Antirrhinum* were considered to be responsible for the pollen S function. However, recent findings in *P. inflata* provide a different scenario in which multiple SLF genes could collaborate to determining jointly the pollen S function in this species.

Kubo *et al.* (2010) extended the competitive interaction experiments initially developed for S_2-SLF to a wider range of SLF genes in *P. inflata*. By sequencing SLF alleles from several S haplotypes, they found that the S_7-SLF was identical to S_{19}-SLF, despite belonging to functionally distinct S specificities. When introduced in appropriate S-heterozygous genotypes, S_7/S_{19}-SLF was found to be able to trigger competitive interaction in heteroallelic pollen when coupled with the S_9 or the S_{17} haplotypes, but not with S_5 or S_{11}. Similarly, other SLF alleles elicited competitive interaction only in pollen of a subset of non-self S haplotypes; the authors argued that despite being indeed involved in the determination of S specificity, SLF could not be the sole pollen S. The *Petunia* S locus contains, besides SLF, a number of additional F-box genes that were previously considered not to be responsible for S specificity; the authors renamed SLF as Type-1 SLF (SLF1), and characterized five more F-box gene types, from Type-2 to Type-6 SLF, foreseeing that they might also function as pollen S. This hypothesis was confirmed by the evidence that, like SLF1, the alleles of SLF2 and SLF3 could also trigger competitive interaction against a subset of S haplotypes: thus, at least three different SLF genes proved to be responsible for the determination of pollen S specificity in *Petunia*.

These findings led the authors to formulate a new model for S-RNase-based SI in *Petunia*, in which each of the F-box proteins encoded within the S-locus contributes to the pollen S function by recognizing and inhibiting (probably through ubiquitination) a single or a subset of non-self-S RNases (Fig. 2). As a result of the concerted action of all the SLFs produced by its S haplotype, the pollen tube would be able to inhibit all the non-self S-RNases; likewise, each functional S haplotype lacks the pollen S function

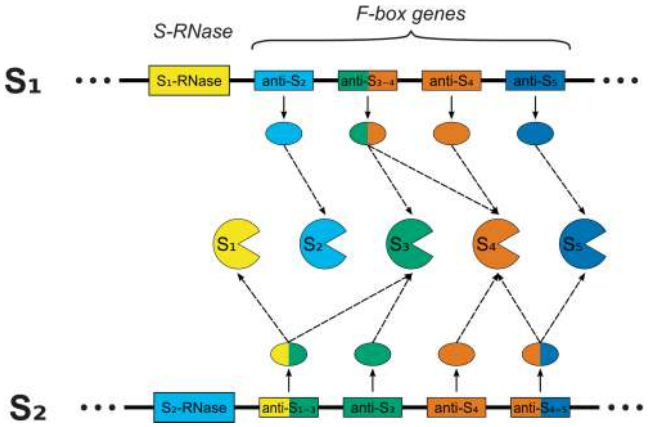

Fig. 2. Schematic diagram of the collaborative non-self recognition model proposed by Kubo *et al*. (2010). The S-locus contains a single pistil S and multiple pollen S genes, whose expression produces the S-RNase (centre) and a pool of F-box proteins with different recognition specificity, respectively; each of the F-box proteins is thus responsible for the recognition and inhibition of one or a few S-RNase alleles (dashed lines), probably by mediating the ubiquitination of the protein. The pool of F-box genes harboured by an S haplotype is sufficient to recognize and inhibit all S-RNases except the self-haplotype. Thus, for example, pollen bearing the S_1 haplotype can inhibit all except the S_1-RNase. It should be noted that, as observed in *Petunia*, different F-box genes within the same haplotype can be targeted to the same S-RNase allele, and different haplotypes (S_1 and S_2 in the figure) can possess different genes recognizing the same S-RNase allele.

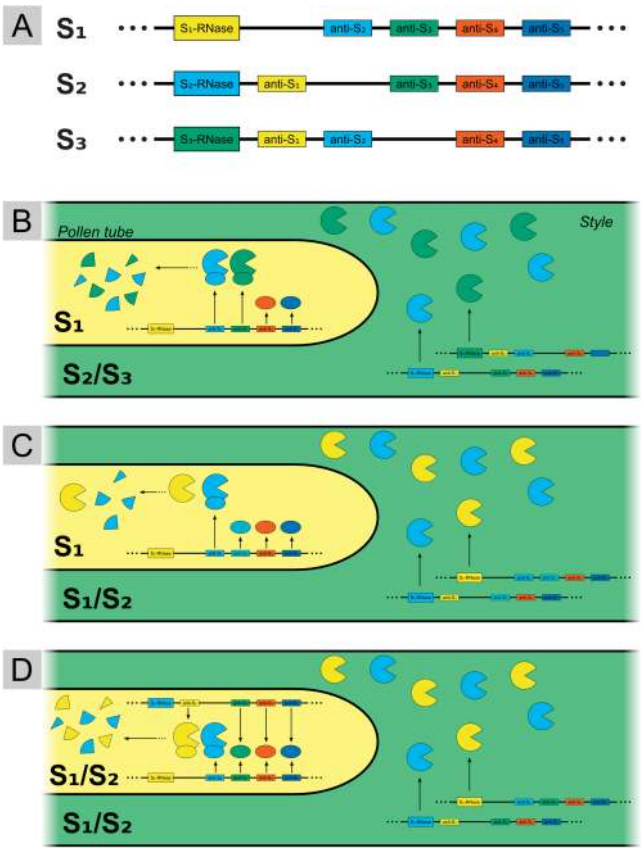

Fig. 3. Mechanism of pollen acceptance and rejection according to the collaborative non-self recognition model. (A) For simplicity we assume in this figure that single F-box genes are responsible for the recognition of single S-RNase alleles; thus each haplotype carries one F-box gene for each of the non-self-S-RNase alleles. (B) Acceptance of S_1 pollen by a S_2/S_3 pistil; the F-box proteins synthesized within the growing pollen tube recognize both S-RNases produced by the pistil and mediate their proteolytic degradation. (C) Rejection of an S_1 pollen tube by a S_1/S_2 pistil; while the non-self S-RNase (S_2, light blue) is degraded, the self S-RNase (S_1, yellow) is left intact and exerts its cytotoxic activity, leading to the death of the pollen tube. (D) Competitive interaction in diploid, heteroallelic S_1/S_2 pollen; as a result of the simultaneous presence of two different S haplotypes, the pollen tube acquires the ability to recognize and degrade all S-RNase alleles, including the self-alleles.

responsible for recognizing its own S-RNase, whose cytotoxic activity would lead to the rejection of self-pollen (Fig. 3). This new model for S-RNase degradation proposed for the *Petunia* GSI system differs quite dramatically from the previous models, as it predicts the pollen S function to be exerted by a number of F-box proteins, rather than a single one. The proposed molecular interaction between female and male S determinants thus extends the inhibitory mechanism proposed for F-box proteins under the previous models: in spite of a single pollen protein recognizing all the 'non-self' S-RNase alleles, it postulates the action of a pool of F-box proteins each having specificity for a subset of S-RNases.

It is intriguing that this model developed for *Petunia* is not consistent with experimental evidence recovered in *Prunus*, suggesting that the SI systems of *Prunus* and *Petunia* may have developed different molecular recognition mechanisms. According to Tao and Iezzoni (2010) the system of *Prunus* and that of the Solanaceae would involve different signal transduction pathways leading to pollen recognition and rejection. This hypothesis is supported by the following experimental evidence: in *Prunus*, loss-of-function SFB in several species is associated with the breakdown of SI leading to self-compatible pollen-part mutants, which provided strong support for the role of SFB as pollen S. In contrast, such types of mutations have never been described in the Solanaceae or Plantaginaceae, neither could pollen-part mutants be obtained after mutagenesis. A likely hypothesis to explain this behaviour was that the pollen S factor is essential for pollen viability or acceptance in these two families (Golz *et al.*, 2001; Xue *et al.*, 2009). Additionally, competitive interaction in polyploids is a widely reported phenomenon in the Solanaceae leading to the breakdown of SI. In contrast, tetraploids in *Prunus* exhibit a different behaviour, as they retain SI when carrying four functional S haplotypes, whereas self-compatibility only arises as a consequence of the accumulation of non-functional S haplotypes (Hauck *et al.*, 2002, 2006).

Altogether these differences suggest that although the SI recognition in both *Prunus* and Solanaceae involves S-haplotype-specific interaction between the S-RNase and SLF/SFB, a key difference must exist in the molecular and

functional consequences of this interaction. Thus in *Prunus*, the self-interaction between SFB and the S-RNase would trigger cytotoxic activity (Tao and Iezzoni, 2010), whereas the non-self interaction of SLF and the S-RNase in the Solanaceae would involve a detoxification effect (Hua *et al.*, 2008; Kubo *et al.*, 2010). Most remarkably, this difference predicts that whereas the S-RNase in the Solanaceae exerts its toxic activity by itself, a compatible interaction in *Prunus* would require the action of some kind of as yet unknown mechanism inhibiting the activity of the S-RNase (Tao and Iezzoni, 2010). This difference is not trivial as it seems to involve the evolutionary divergence of the two recognition mechanisms that may have originally been the same. Alternatively, *Prunus* and Solanaceae SI could represent a similar but not identical mechanism with different origins, representing a case of evolutionary convergence.

The phylogenetic reconstruction of S-RNases and SLF/SFB/SFBB in the three families with S-RNase-based GSI, Rosaceae, Solanaceae, and Plantaginaceae, might shed some light on this question. Thus, the tree of the S-RNases supports a monophyletic origin of the gene in the three families (Fig. 4A), suggesting that the gene and hence the system probably evolved only once, early in the evolutionary history of Eudicots (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002; Vieira *et al.*, 2008*a*). In contrast, SLFs from Solanaceae and Plantaginaceae seem to be more closely related to Pyrinae SFBBs than to SFBs from *Prunus* (Fig. 4B) which have been characterized as a divergent group with an uncertain origin in the phylogenetic context of the superfamily of S-locus F-box-like proteins of a wide range of species (Vieira *et al.*, 2009). One explanation for this evolutionary pattern would be that the closer phylogenetic relationship between SLFs and SFBBs may reflect a conserved ancestral mechanism for the pollen S function of Solanaceae and Pyrinae, whereas the *Prunus* SI system could have diverged by developing a different mechanism of self/non-self pollen recognition (Tao and Iezzoni, 2010). Although it is an interesting hypothesis, its confirmation would need further clarification of the actual mechanism controlling the pollen S function of Pyrinae. Interestingly, the *Prunus* S-locus region contains additional F-box genes besides SFB, named SLFL for S-locus F-box genes with low allelic polymorphism (Entani *et al.*, 2003; Ushijima *et al.*, 2003); these genes are not known to play a role in the pollen S function, but they show the highest homology to the Pyrinae SFBBs (Fig. 4B; Sassa *et al.*, 2007; Matsumoto *et al.*, 2008), supporting the hypothesis that the common ancestor of *Prunus* and the Pyrinae could have contained multiple F-box genes within its S-locus region.

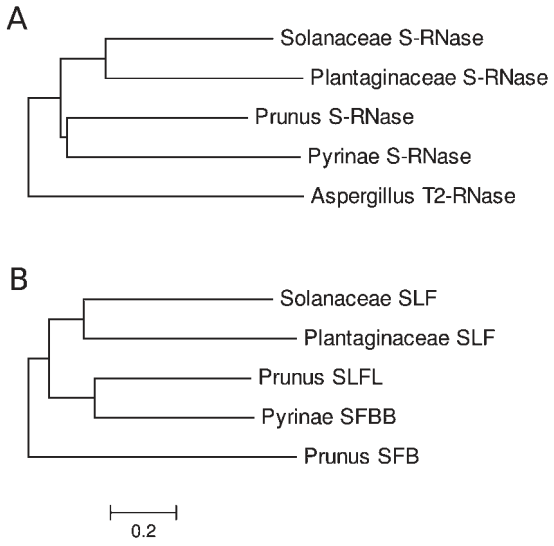

Fig. 4. Neighbor–Joining tree of the S-locus genes from the different taxa possessing S-RNase-based GSI. (A) The phylogenetic analyses carried out on the S-RNase sequences support a monophyletic origin for this gene. (B) The Pyrinae SFBBs are more closely related to Solanaceae and Plantaginaceae SLFs than to *Prunus* SFBs; nevertheless, the *Prunus* S locus harbours additional genes (SLFLs) not involved in the determination of S specificity, which cluster together with SFBBs.

At present, limited functional information exists about the pollen S determinant of Pyrinae; however, several lines of evidence suggest that the Pyrinae could exhibit an SI recognition system more related to that of the Solanaceae than to the SI system of *Prunus*. Thus, similarly to the Solanaceae, the Pyrinae exhibit competitive interaction in tetraploids (Lewis and Modlibowska, 1942; Adachi *et al.*, 2009; Qi *et al.*, 2011). Moreover, no loss-of-function pollen S mutants leading to self-compatibility have been reported; in Chinese pear a pollen-part mutation has been analysed by Li *et al.* (2009), but it has not yet been characterized at the molecular level, so it might possibly depend on a duplication, rather than a deletion, of pollen S genes, as described in *Nicotiana* and *Antirrhinum* (Golz *et al.*, 1999, 2001; Tsukamoto *et al.*, 2005; Xue *et al.*, 2009). Finally, the SI behaviour of the self-compatible haplotype of Japanese pear, S_4^{sm}, has been proposed to be consistent with the predicted behaviour of loss-of-function single F-box genes, under the collaborative non-self-recognition model proposed for *Petunia* (Kubo *et al.*, 2010). Okada *et al.* (2008) characterized the deletion within the S_4^{sm} haplotype of 'Osa-Nijisseiki', a mutation of the S_4 haplotype of 'Nijisseiki' (Sassa *et al.* 1997) that confers self-compatibility; the deleted region, consisting of 236 kb, was fully sequenced from a BAC library prepared from an S_4 homozygote, selected from a bud-selfed progeny of 'Nijisseiki'. Consistently with the stylar-part nature of such mutation, the deletion included the S_4-RNase, but also the F-box gene placed immediately downstream (named S_4-Fbox0). Interestingly, as a result of this deletion S_4^{sm} pollen is incompatible not only in S_4 styles but also in styles expressing the S_1 specificity (Okada *et al.*, 2004; Kakui *et al.*, 2011; Saito *et al.*, 2012). Thus, according to this evidence, Kubo *et al.* (2010) predicted that S_4-Fbox0 could be an element of the pollen S in *Pyrus pyrifolia* specifically recognizing the S_1-RNase (Fig. 5).

If this hypothesis is correct, then the collaborative recognition system would have been maintained in members of the distant families Rosaceae and Solanaceae. Therefore, it most probably would correspond to the ancestral recognition system of S-RNase-based GSI, from which the

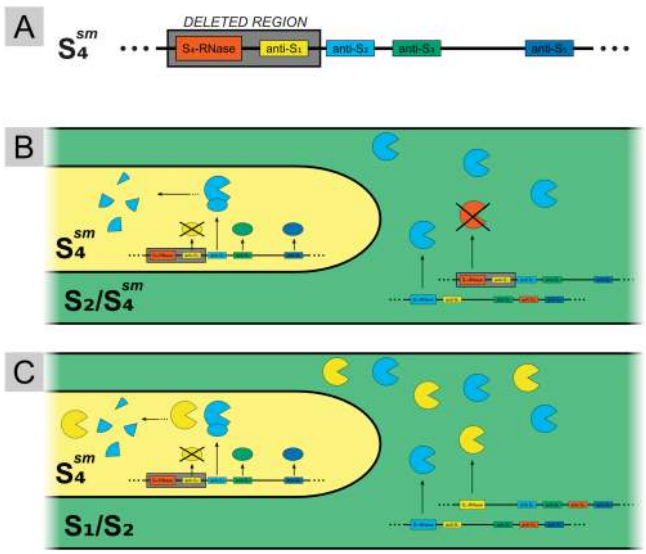

Fig. 5. Behaviour of the S_4^{sm} haplotype of Japanese pear (Okada *et al.*, 2008), according to the collaborative recognition mechanism (Kubo *et al.*, 2010). (A) With respect to the original S_4, the mutant S_4^{sm} haplotype lacks a 236 kb region including the S_4-RNase and one F-box gene, named S_4-Fbox0, which is thought to be responsible for the specific inhibition of the S_1-RNase. (B) Style-part self-compatibility conferred by the S_4^{sm} haplotype is due to the lack of the S_4-RNase (orange). (C) Rejection of S_4^{sm} pollen by pistils carrying the S_1 haplotype. The deletion in S_4^{sm} of the F-box gene responsible for the recognition of S_1-RNase impairs inhibition of the S_1-RNase and causes rejection of the non-self S_4^{sm} pollen tube.

Prunus system might have diverged at some point during evolution. How this transition occurred is not easy to explain, given that, according to the existing biochemical models, it would have implied a change in the role of the pollen S: while in *Petunia* SLF proteins are supposed to act as inhibitors of the S-RNase, in *Prunus* SFB probably elicits its function by protecting the S-RNase from inhibition by an as yet uncharacterized mechanism, perhaps protein degradation triggered by non-polymorphic SLFLs (Matsumoto *et al.*, 2008). Thus, SFB would have acquired a function that is the opposite to the ancestral one. One possibility is that this shift occurred in two distinct stages, the first involving the breakdown of the ancestral GSI system through the acquisition of a general inhibitor for S-RNases; and the second restoring GSI through the recruitment of an S-locus gene to protect the S-RNase from this general inhibitor. At present, however, no experimental evidence is available to address this hypothesis. Nevertheless, it seems reasonable to hypothesize that S-RNase-based GSI evolved via different paths within the family Rosaceae: the Pyrinae system, as summarized in Table 1, seems to resemble the ancestral mechanism as observed in the Solanaceae, whereas the *Prunus* GSI features indicate the existence of at least one different mechanism within this family. Further variants of the S-RNase-based GSI mechanism could moreover exist in other Rosaceae, one of which might be the genus *Fragaria*, in which SI seems to be controlled by two independent S loci (Bošković *et al.* 2009).

The mechanism of incompatible pollen rejection

The degradation of cellular RNA is considered in itself an event sufficient to cause the death of the cell (McClure *et al.*, 1989); therefore, after the discovery that the pistil S determinant of *Nicotiana* had RNase activity, and the demonstration that this activity was required for pollen rejection (Huang *et al.*, 1994), it was hypothesized that S-RNases might have a direct cytotoxic effect, and that RNA degradation was necessary and sufficient to inhibit the growth of incompatible pollen tubes. Consequently, more effort was made to discover the mechanism of S-RNase detoxification in compatible pollen, rather than to investigate the cytotoxic effect in incompatible tubes. However, it is possible that pollen rejection could occur through a mechanism that is triggered, but not directly executed, by the S genes. This hypothesis is supported by the evidence that *N. alata* pollen tubes challenged with self S-RNases are capable of recovering from inhibition if the top of the incompatible pistil is grafted on a compatible one (Lush and Clarke, 1997); thus, the mechanism of pollen rejection is reversible at least in its initial phase, whereas a direct cytotoxic action of self S-RNases would most probably be irreversible.

Recent findings in the Pyrinae have contributed to shedding some light on the mechanism of pollen rejection (Fig. 6); experiments monitoring the events occurring in *P. pyrifolia* pollen tubes exposed to self S-RNase revealed a dramatic rearrangement of the cytoskeleton, with actin depolymerization and the formation of high molecular mass aggregates (Liu *et al.*, 2007). Moreover, the collapse of the mitocondrial membrane potential, with consequent leakage of cytochrome *c* into the cytosol and ROS (reactive oxygen species) disruption, and nuclear DNA degradation (Wang *et al.*, 2009, 2010) also seem to be phenomena associated with the cytotoxic activity of S-RNases (Fig. 6B). All these events are characteristics of programmed cell death (PCD) and suggest that incompatible pollen inhibition in the Pyrinae might have some common features with that of poppy (*Papaver rhoeas*). The Papaveraceae possess a distinct GSI system, in which the S locus encodes a male and a female determinant acting as a cell surface receptor and a protein ligand, respectively (Foote *et al.*, 1994; Wheeler *et al.*, 2009). In this system, self-pollen rejection occurs through PCD and involves at least part of the cellular modifications which were also reported in *Pyrus*: actin depolymerization, mitocondrial alteration, and nuclear DNA degradation (Thomas and Franklin-Tong, 2004). The induction of PCD in *Papaver* pollen is mediated by a mitogen-activated protein kinase (MAPK) cascade initially triggered by a rapid increase in the cytosolic free calcium concentration ($[Ca^{2+}]_i$). A signal cascade has also been proposed in *P. pyrifolia* (Wang and Zhang, 2011) in which the intracellular calcium might play a crucial role as well, since a decrease of the Ca^{2+} current was observed in incompatible pollen (Wang *et al.*, 2010), even though the effect of self and non-self S-RNases on the $[Ca^{2+}]_i$ was unclear (Xu *et al.*, 2008).

Table 1. Comparison between the S-RNase-based GSI systems of *Petunia*, *Prunus*, and the Pyrinae

Taxon	Pistil S	Pollen S	Supposed action of pollen S	Competitive interaction	Frequency and nature of PPM	Effect of pollen S gene(s) deletion
Petunia	S-RNase	Multiple F-box proteins	Inhibition of non-self S-RNases	Yes	Extremely rare, duplication of pollen S genes	Extended pollen cross-incompatibility (supposed)
Prunus	S-RNase	Single F-box protein	Protection of self S-RNase from inhibition	No	Quite frequent, inactivation of pollen S gene	Pollen-part self-compatibility
Pyrinae	S-RNase	?	?	Yes	Extremely rare	Extended pollen cross-incompatibility

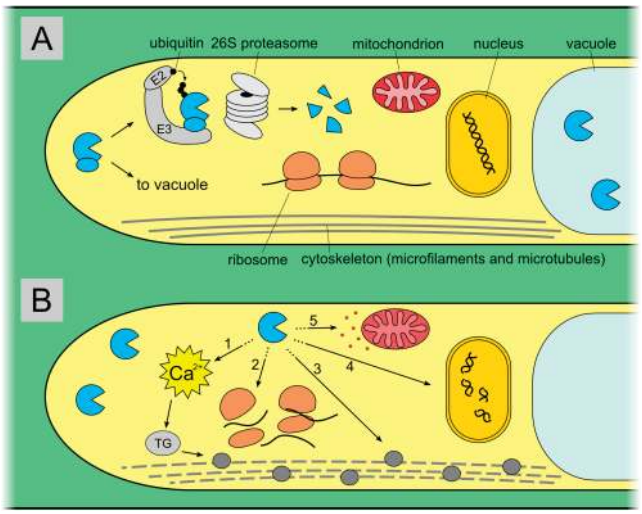

Fig. 6. Proposed biochemical and cytological events underlying the acceptance or rejection of pollen tubes. (A) Acceptance: the non-self S-RNases, once imported inside the pollen tube, are specifically recognized by F-box (SFBB) proteins, which are thought to participate in an E3 ubiquitin ligase enzymatic complex. The S-RNase is thus ubiquitinated and directed to proteolytic degradation by the 26S proteasome; alternatively, another mechanism to prevent the S-RNase cytotoxicity might be its compartmentalization in the vacuole. (B) Rejection: the self S-RNase cannot be recognized due to the lack of a specific F-box protein, and can thus exert its activity finally leading to the death of the pollen tube. The cellular response to the self S-RNase involves a modulation of the intracellular calcium concentration (1), that might be the first step in the signalling pathway. The RNase activity of the S-RNase is supposed to mediate the degradation of rRNA (2). Later events include a rearrangement of the cytoskeleton, with actin depolymerization and the formation of high molecular mass aggregates (3), possibly mediated by the cross-linking activity of the calcium-dependent enzyme transglutaminase (TG); the release of cytochrome *c* from mitochondria (4); and nuclear DNA degradation (5). These later events suggest that the inhibition of incompatible pollen tubes finally occurs through programmed cell death (PCD).

A recent study in European pear suggested that polyamines and the enzyme transglutaminase (TGase) could also play a role in mediating the response to self/non-self pollen recognition (Del Duca *et al.*, 2010). TGase is a calcium-dependent enzyme that catalyses the covalent binding between glutamyl residues of proteins and amine donors, such as lysyl residues or polyamines, forming cross-links; they are thus responsible for the formation of bridges between specific proteins, and proved to be involved in a wide variety of cellular mechanisms, including the regulation of cell growth and differentiation. In pistils pollinated with incompatible pollen, the content of the three main polyamines (putrescine, spermine, and spermidine) was found to be lower than in styles pollinated with compatible pollen; in contrast, the TGase activity was higher. In apple pollen tubes, actin and tubulin are substrates of the TGase and its activity is responsible for the formation of high molecular mass aggregates of actin, suggesting an involvement of this enzyme in the regulation of cytoskeleton assembly and dynamics (Del Duca *et al.*, 2009). Since TGase is a calcium-dependent enzyme, it has been proposed that an increase in $[Ca^{2+}]_i$ in incompatible pollen tubes could be responsible for the up-regulation of its activity (Fig. 6B), and thus for the formation of the actin foci that have been observed during pollen rejection (Liu *et al.*, 2007). Both polyamines and TGase have been proposed as modulators of PCD, with mechanisms that seem to be at least in part conserved with animal cell apoptosis (Della Mea *et al.*, 2007). The observed variation in polyamine content and TGase activity can thus be considered an additional clue supporting PCD as a mechanism of pollen rejection. Intriguingly, polyamines are also known for their inhibitory effect on RNases. Thus, the increase in polyamine concentration observed in styles after compatible pollination has been proposed as a possible mechanism involved in the inhibition of non-self S-RNases (Del Duca *et al.*, 2010).

Several clues thus suggest that the recognition of incompatible pollen involves a cascade of events leading to PCD; this cascade is triggered by the specific interaction between the male and female S determinants, but so far it remains unknown how self-S-RNases would act in the initiation of a putative PCD cascade of events or in its signalling pathway. Even though the RNase activity proved to be necessary for pollen rejection, the S-RNase may have additional functions, such as the induction of cell death, similar to some T2-type RNases in other organisms: it is

thus unclear whether the degradation of rRNA is a cause or a consequence of the self-pollen inhibition mechanism (Chen *et al.*, 2010; Matsumoto and Tao, 2012).

The mechanism of detoxification of S-RNases in compatible pollen also needs to be further elucidated; several components of an E3 ubiquitine ligase complex putatively involving S-locus-encoded F-box proteins have been identified, suggesting that S-RNase is ubiquitinated after the specific interaction with the male determinant (Fig. 6A), even though direct *in vivo* evidence is still needed to confirm this hypothesis (reviewed in Zhang *et al.*, 2009); one of the putative components of this complex, named AhSSK1 (*Antirrhinum hispanicum* SLF-interacting Skp1-like1), interacting with both SLF and cullin, was identified in *Antirrhinum* (Huang *et al.,* 2006), and its *Petunia hybrida* equivalent PhSSK1 proved to be required for compatible pollen acceptance (Zhao *et al.*, 2010). Non-self S-RNases might thus be targeted to the 26S proteasome, and their proteolytic degradation could be the required event for compatible pollen tube acceptance. However, a different mechanism has also been proposed; in *Nicotiana*, S-RNases proved to be sequestered in vacuoles after their uptake, being subsequently released in the cytosol only in incompatible pollen tubes (Goldraij *et al.*, 2006). S-RNase compartmentalization might thus prevent their cytotoxicity, and their release would occur as the pollen tube is rejected. Even in this case, however, it is not yet possible to state whether the S-RNase release is the cause or a consequence of pollen inhibition. It is noteworthy that the rupture of the tonoplast membrane and the consequent release of the vacuolar content in the cytosol is one of the events occurring during PCD in plants (Della Mea *et al.*, 2007).

Evolution of the Pyrinae S-locus

Genetic models of SI typically assume single genes for the male and the female functions, whose self-recognition triggers the SI response. This is, for instance, the case of sporophytic SI of Brasicaceae, GSI of Papaveraceae, or even of GSI of *Prunus* which is also based on the S-RNase system (Takayama and Isogai, 2005). Under this model, since the creation of a new S function involves the maintenance of self-recognition within S haplotypes, coordinate changes between the pistil and the pollen S genes have been invoked and the pollen S is assumed to have co-evolved with the pistil S. However, as Newbigin *et al.* (2008) pointed out, *Petunia* and *Antirrhinum* SLFs showed much lower levels of sequence polymorphism than their corresponding S-RNases, and no or little support was found for positive selection acting on them. Likewise, low variability and little evidence of positive selection were found in several Pyrinae SFBBs (De Franceschi *et al.*, 2011*a*). The initial conclusion was that the genes considered as the best candidates for the role of pollen S did not show, at least in the Solanaceae, *Antirrhinum*, and the Pyrinae, all the expected features for the role they were supposed to play, suggesting that either they had been falsely identified or there were major problems with our understanding of how pollen S evolves (Newbigin *et al.*, 2008; Vieira *et al.*, 2009). These incongruities, however, could be explained at least in part according to the collaborative non-self recognition system of Kubo *et al.* (2010) which involves multiple F-box genes as the pollen S, whose interaction with non-self S-RNases mediates a compatible response rather than the SI response assumed for single-gene pollen S models. Interestingly, the patterns of sequence diversity and the phenotypic behaviour of loss-of-functions mutants of Pyrinae seem to be consistent with this new model of self-recognition (De Franceschi *et al.*, 2011*a*; Kakui *et al.*, 2011).

The large number of related F-box genes lying at the S-locus region of species of the Solanaceae, Plantaginaceae, and Pyrinae suggests that gene duplication could have been an active process allowing proliferation of SLF/SFBB genes, and the functional diversification of the pollen S functions (Kubo *et al.*, 2010; Minamikawa *et al.*, 2010; De Franceschi *et al.*, 2011*a*). As Kubo *et al.* (2010) predicted under the collaborative non-self recognition model, increasing the repertoire of F-box genes can be advantageous for S haplotypes as it would increase the number of potential mating partners by allowing the pollen to detoxify more non-self S-RNases. Evidence provided by Kubo *et al.* (2010) and Kakui *et al.* (2011) suggests that multiple genes encode the pollen function in the Solacaceae and the Pyrinae; moreover, pollen S genes can exhibit dual or multiple recognition specificities (Kubo *et al.*, 2010). Thus, according to these results one could envisage two modes by which an S haplotype could acquire a new pollen S function. Mutation of an existing SLF/SFBB could be a mechanism, if the gene acquires a new pollen S function while it preserves its former functionality. An existing SLF/SFBB could also undergo gene duplication, allowing mutation and functional diversification of the duplicated gene without affecting the state of the original gene repertoire. It is likely, however, that the second possibility is favoured over the first one (Kubo *et al.*, 2010): if each F-box gene is responsible for the recognition of one or a few S-RNase alleles, a mutation modifying the function of one of them could result in the inability to recognize its former 'target' S-RNase(s), thus reducing the fitness of the S haplotype. Therefore, selection would tend to eliminate these mutational events from the population. In contrast, duplication of an F-box gene should not necessarily alter the male S function, so such an event might have no phenotypic effect at all, or might have a positive effect if an increased gene dosage allows a more efficient recognition and inhibition of its target S-RNase(s). Thus, duplication may favour the diversification potential of S haplotypes for new pollen S functions, provided that mutation of the duplicated gene would allow functional diversification, while the original copy preserves the pollen S functionality. In agreement with these assumptions, SFBB duplication and subsequent diversification seem to have accompanied the evolution of the S locus of Pyrinae.

It is intriguing how this pattern of pollen S genes evolution may fit with the function and pattern of diversification of the single pistil S gene. According to Kubo

et al. (2010) diversification of the S-RNase towards a new pistil S function would be disadvantageous for the S haplotype as the new S-RNase would escape detoxification by the whole repertoire of existing pollen S functions, resulting in female sterility; such a mechanism would thus compromise the proliferation and maintenance of any new pistil S function in the population. We argue, however, that such rare events, although being selectively compromised, could provide a suitable environment for the selection of S haplotypes with the potential of diversification (i.e. bearing an SLF/SFBB gene duplication), given that the development of a new pollen S function by the duplicated SLF/SFBB targeting the new S-RNase would break the sterility of the plant bearing the new pistil S function (Fig. 7). The S haplotype with the new pollen S function would have a higher chance of mating with the plant having the new pistil S function, given that this would be the only fertile combination; thus, the novel pollen S function would confer an increased fitness to its S haplotype. Spreading of the new SLF/SFBB among other S haplotypes through single gene duplication events could be thus favoured by selection: ideally, mechanisms such as retroposition or retrotransposon-mediated gene duplication (Xiao *et al.*, 2008; Flagel and Wendel, 2009) can allow a horizontal gene transfer between S haplotypes in diploid cells; such an event would be favoured when the receiving haplotype acquires the ability to degrade the novel S-RNase allele, resulting in an increased pollen fitness. This pattern of F-box gene proliferation could shed some light on why different S haplotypes exhibit such different S-locus structures with different gene ordering.

This mode of diversification of S haplotypes would leave an imprint on the pollen S genes that may be consistent with the S-locus structure and the pattern of diversification and phylogenetic structure of *Petunia*/*Antirrhinum* SLFs, or with the Pyrinae SFBBs. The unexpected features of pollen S candidates, namely a low degree of sequence polymorphism and absence of positive selection (Newbigin *et al.*, 2008; De Franceschi *et al.*, 2011*a*), came from the comparison of those *Petunia* SLFs that later proved to be genes related to one another and responsible for the recognition of the same S-RNase allele(s) (Type-1 SLF; Kubo *et al.*, 2010). Even though no direct experimental evidence is yet available for the collaborative model in the Pyrinae, the existence of multiple pollen S genes seems to be in agreement with extant phylogenomic data. When analysing related SFBBs of different S-haplotypes, lack of positive selection seems to be the rule (De Franceschi *et al.*, 2011*a*); indeed according to the collaborative non-self recognition system, one should expect a role for purifying selection in preserving the inhibitory activity of the SFBBs. Variation, in contrast, would be favoured on duplicated genes, since it would be the basis for the recognition of new S-RNase alleles; consistent with this hypothesis, support for positive selection was found for one of the Pyrinae F-box genes (SFBBβ), which exhibited events of gene duplication in several haplotypes (De Franceschi *et al.*, 2011*a*).

Based on this reasoning, the low degree of polymorphism and the absence of positive selection reported for S-locus F-box genes should not be considered inconsistent with their involvement as pollen S. On the contrary, one could argue that their high degree of conservation and evidence of purifying selection indicate that they are derived from a common ancestor and have been maintained by selection, because they are responsible for the inhibition of the same S-RNase allele(s); this could be considered indirect evidence of the collaborative recognition model also operating in the Pyrinae S locus. Thus, the different phylogenetic profiles of SFBBs and the S-RNase are likely to reflect different evolutionary histories, with SFBBs being much younger than the S-RNase, as well as different evolutionary modes (De Franceschi *et al.*, 2011*a*). Moreover, it is worth noting that functional diversification of the S-locus genes, including the S-RNase, should not necessarily involve single transitional events; in contrast diversification could involve gradual transitions exhibiting a repertoire of intermediate phenotypes. In the European pear, recent evidence suggests that neutral variants of the S-RNase can co-exist within the species, highlighting that diversification of the S-RNase in the Pyrinae is an ongoing process, and that the protein exhibits enough plasticity to undergo sequence divergence while preserving functional specificity (Sanzol, 2010). Ideally, neutral evolution for long evolutionary periods could favour a concerted evolution of the S-RNase and SFBBs.

Significantly, the only pollen S candidates that in previous studies highlighted levels of sequence polymorphism similar to the S-RNase and evidence for positive selection were the *Prunus* SFBs (Nunes *et al.*, 2006; Newbigin *et al.*, 2008; Vieira *et al.*, 2008*b*); this is in agreement with the different recognition mechanism that is believed to characterize the *Prunus* GSI, in which there is specificity between a single pistil and a single pollen S gene.

Conclusions and future prospects

The last two decades have produced many studies directed at unravelling the molecular basis of the SI system of Pyrinae. Much of the effort by the scientific community has been concentrated on the molecular characterization of the S-locus region as it contains the genes for the female and the male functions of self-recognition. The characterization of the S-RNase as the pistil S allowed an initial approximation to address some important questions regarding the genetics, biochemistry, and evolutionary biology of SI in this group. This achievement has also facilitated important advancements in applied research, by allowing the development of molecular S-genotyping methods, the characterization and incorporation in breeding programmes of self-compatible mutations, and laying the ground for engineering this trait of significant agricultural relevance. Similarly, as we stress throughout this manuscript, research in the Pyrinae has also contributed to our general understanding of S-RNase-based GSI, so it is expected that future research in this taxonomic group will contribute in the same way to this important field of plant biology.

At present, the main effort of several laboratories is concentrated on uncovering the gene(s) responsible for the

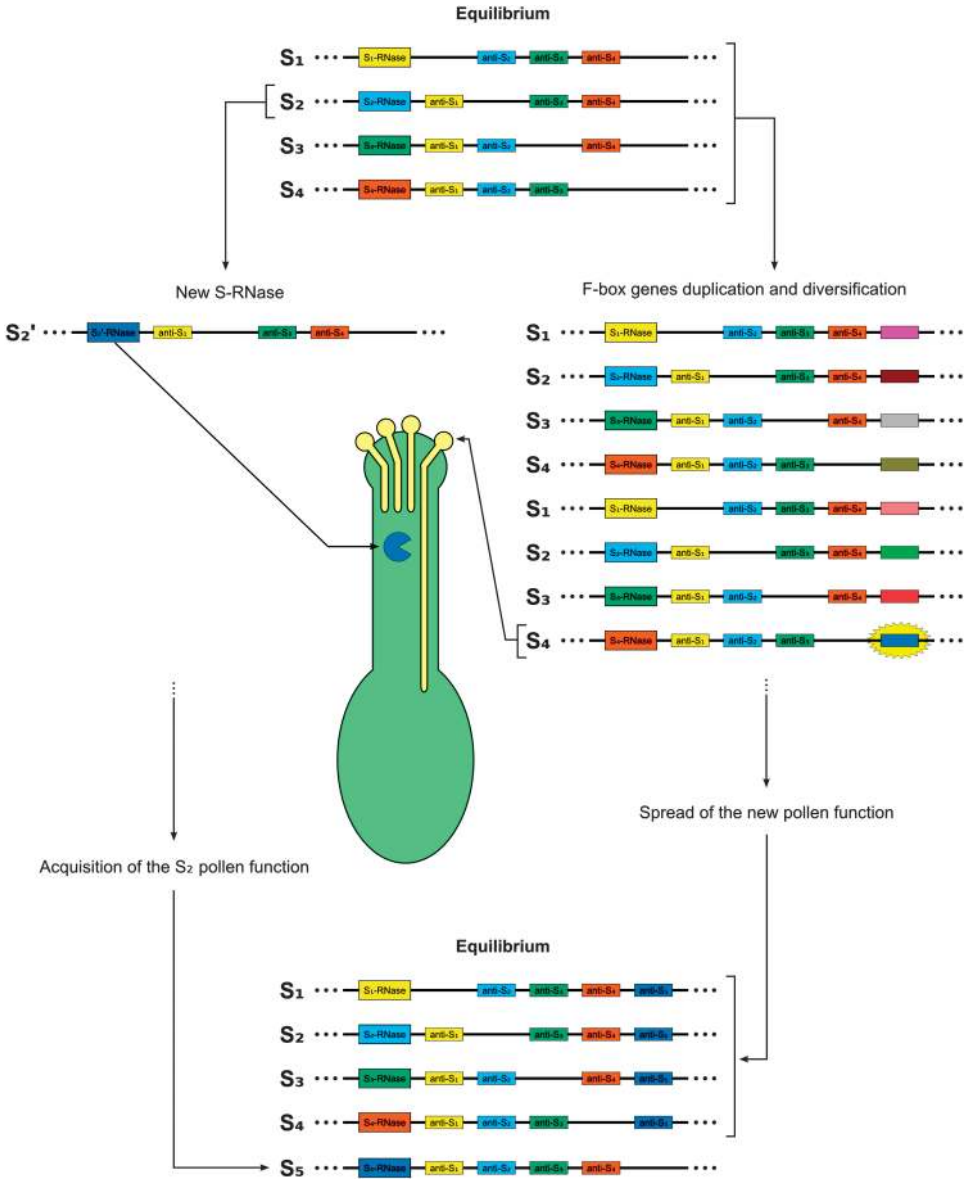

Fig. 7. Coordinate changes in S haplotypes leading to the generation of a new specificity. In the upper part of the figure is represented an ideal equilibrium between four S haplotypes: each of them has enough F-box genes to recognize all the non-self S-RNases. According to the collaborative recognition model, changes of the pistil and pollen S genes required for generating new specificities are expected to occur in distinct haplotypes. On the left, one or several mutations on the S-RNase of the S_2 haplotype results in S_2', a new pistil-side specificity. The newly generated S-RNase allele cannot be recognized by any of the F-box proteins produced by the existing S haplotypes; therefore, a pistil expressing the S_2'-RNase (in the centre) cannot be fertilized by pollen bearing the pool of S haplotypes in the population and it behaves as female-sterile. On the right side, duplication and subsequent mutation of F-box genes allows the diversification of pollen S functions within haplotypes. Among all the randomly generated S haplotypes having new pollen S genes, the one that accidentally matches with the S_2'-RNase will confer the ability to fertilize the plant with the S_2'-RNase. Thus, the presence of the new S-RNase allele will favour the fixation of the pollen-side mutated haplotype in the population; conversely, the presence of the new pollen-side specificity will allow the pistils expressing the new S-RNase to escape female-sterility. A new equilibrium will be reached when all the S haplotypes in the population have acquired the ability to recognize and degrade all except the self S-RNase: thus, S_2' must acquire the S_2 pollen function whereas all the others S-haplotypes must acquire the S_2' pollen function. At this point, S_2' can be considered a fully functional new S specificity (S_5).

male function. So far, several genes have been characterized in the genomic region surrounding the S-RNase and some of them seem to be good candidates to develop this function. Clarifying their role will also help in understanding the mechanism by which SI in the Pyrinae elicits self/non-self pollen recognition. The data available so far suggest that the *Petunia* and the Pyrinae GSI systems exhibit significant similarities, while the *Prunus* system seems to be a related but divergent mechanism. It has been hypothesized that the collaborative model developed for

Petunia could be extended to the Pyrinae (Kubo *et al.*, 2010; Kakui *et al.*, 2011). If this is correct it could provide an answer to the question first raised by Sassa *et al.* (2007), of whether one or more F-box genes contribute to the pollen S function of this group. If the same molecular mechanism of recognition is maintained between the Solanaceae and the Pyrinae, this might be considered to be the most probable ancestral mechanism of S-RNase-based GSI, from which the *Prunus* ancestors diverged at some point during the evolutionary history of the Rosaceae. However, experimental evidence is needed to confirm that multiple SFBB genes act together as pollen S in the Pyrinae, and thus to support the applicability of the collaborative non-self recognition model to this taxon; the transgenic approach that was successfully applied to the study of *Petunia* SLFs is hampered by the woody habit of these species. An alternative approach may rely on *in vitro* experiments, such as the analysis of protein–protein interactions, which could be applied to SFBBs in order to test their ability to bind the non-self S-RNase alleles specifically.

Furthermore, some aspects remain unclear regarding the supposed similarity between the Pyrinae and *Petunia* GSI systems. Several non-S genes have been characterized in the Solanaceae that play a role in SI (reviewed by Chen *et al.*, 2010); the loss or reduced expression of some of them, such as HT-B (H-Top Band) and 120K (120 kDa glycoprotein), results in the breakdown of SI. However, no homologues of these genes have been identified so far in the Pyrinae. Thus, it is possible that some relevant differences exist between the two systems that still need to be investigated, as well as the exact nature of the cytotoxic action of the S-RNase and its mechanism of detoxification.

The proposed involvement of multiple SFBBs in the pollen S function could provide an explanation for the complex structure of the S locus of Pyrinae. The multiplicity of SFBBs within the S haplotypes of Pyrinae would be consistent with the requirement for multiple pollen S genes to allow recognition of a wide range of non-self S-RNases. Future studies should address this hypothesis, which in turn will contribute to understanding what the collaborative recognition system implies regarding the evolutionary dynamics controlling the increase in and differentiation of new S specificities. It seems likely that SFBB gene duplication has been a major force driving the evolutionary dynamics of the S locus, and it may have been a source of genetic material allowing functional diversification of the S locus. Finally, further work will be needed to ascertain the mechanism operating downstream of pollen–pistil S gene recognition. PCD may be a mechanism in incompatible pollen rejection, but other signalling cascades have been proposed to underly this activation.

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