How flowering plants discriminate between self and non-self pollen to prevent inbreeding

Teh-hui Kao, and Andrew G. McCubbin

PNAS 1996;93;12059-12065
doi:10.1073/pnas.93.22.12059

This information is current as of November 2006.
How flowering plants discriminate between self and non-self pollen to prevent inbreeding

TEH-HUI KAO AND ANDREW G. MCCUBBIN

Department of Biochemistry and Molecular Biology, 403 Althouse Laboratory, Pennsylvania State University, University Park, PA 16802

ABSTRACT Flowering plants have evolved various genetic mechanisms to circumvent the tendency for self-fertilization created by the close proximity of male and female reproductive organs in a bisexual flower. One such mechanism is gametophytic self-incompatibility, which allows the female reproductive organ, the pistil, to distinguish between self pollen and non-self pollen; self pollen is rejected, whereas non-self pollen is accepted for fertilization. The Solanaceae family has been used as a model to study the molecular and biochemical basis of self/non-self-recognition and self-rejection. Discrimination of self and non-self pollen by the pistil is controlled by a single polymorphic locus, the S locus. The protein products of S alleles in the pistil, S proteins, were initially identified based on their cosegregation with alleles. S proteins have recently been shown to indeed control the ability of the pistil to recognize and reject self pollen. S proteins are also RNases, and the RNase activity has been shown to be essential for rejection of self pollen, suggesting that the biochemical mechanism of self-rejection involves the cytotoxic action of the RNase activity. S proteins contain various numbers of N-linked glycans, but the carbohydrate moiety has been shown not to be required for the function of S proteins, suggesting that the S allele specificity determinant of S proteins lies in the amino acid sequence. The male component in self-incompatibility interactions, the pollen S gene, has not yet been identified. The possible nature of the pollen S gene product and the possible mechanism by which allele-specific rejection of pollen is accomplished are discussed.

Because of the close proximity of the anther and pistil in a bisexual flower, there is a great tendency for pollen to land on the stigma of the same flower. If there were no mechanism to prevent fertilization by self pollen, inbreeding would result, which reduces the genetic variability in the species. Fortunately, this is not the case. A number of strategies have evolved in flowering plants that prevent self-fertilization. One of the strategies is called self-incompatibility. It was described by Charles Darwin in a book published more than a century ago (1). He observed that some plant species were completely sterile to their own pollen, but fertile with that of any other individual of the same species. Since Darwin's observation, self-incompatibility has been found to occur in more than half of the flowering plant species (2).

Self-incompatibility allows the pistil of a flower to distinguish between self (genetically related) pollen and non-self (genetically unrelated) pollen. Self pollen is rejected, whereas non-self pollen is accepted for fertilization. As Darwin observed, self-incompatible plants are completely sterile with respect to self pollen, but fertile with respect to non-self pollen. Self-incompatibility can be classified into homomorphic and heteromorphic types. In the homomorphic type, flowers of the same species have the same morphological type, whereas in the heteromorphic type, flowers of the same species can have two or three different morphological types, and pollination is compatible only between flowers of different morphological types (2). The homomorphic type is further classified into gametophytic and sporophytic types based on whether the pollen behavior in self-incompatibility interactions is determined by the genotype of the pollen itself (gametophytic) or by the genotype of the plant from which the pollen is derived (sporophytic). The gametophytic type is more common (found in more than 60 families of flowering plants) than the sporophytic (found in 6 families), and the two types apparently evolved independently. To date, only one of the sporophytic families (Brassicaceae) and five of the gametophytic families (Papaveraceae, Poaceae, Rosaceae, Scrophulariaceae, and Solanaceae) have been studied at the molecular level (for a recent comprehensive treatise of self-incompatibility, see ref. 3). This article will focus on recent studies carried out on the Solanaceae family, in particular the species Petunia inflata, which has been studied in the authors' laboratory.

Genetic Basis of Self-Incompatibility

In the solanaceous type of self-incompatibility, a single polymorphic genetic locus called the S locus determines the outcome of pollination (see Fig. 1). If a plant carries S1 and S2 alleles, the pollen produced will carry either the S1 or S2 allele. When these pollen grains land on the stigma of the same flower, they will germinate and grow down into the style; however, their growth will be arrested in the upper one-third segment of the style. This is because the pistil recognizes S1 and S2 pollen as self pollen through the matching of the S alleles. The tip of a self pollen tube usually swells and bursts open. These self pollen tubes are thus unable to deliver their sperm cells to the ovary for fertilization. If S2 and S3 pollen from another plant land on the stigma of the S1S2 plant, the result will be different. Again, the S2 pollen will be rejected because of the matching of S alleles. But the S3 pollen, carrying an S allele different from the two carried by the pistil, will be recognized as non-self pollen and will germinate and grow all the way down through the style to the ovary to effect fertilization.

Identification of S Allele-Associated Proteins in Pistil

There are two fundamental questions one can ask about self-incompatibility: (i) How does a pistil distinguish between self and non-self pollen? (i.e., what is the molecular basis of self/non-self discrimination?). (ii) How does self-recognition lead to growth arrest of pollen tubes? (i.e., what is the biochemical mechanism of self-rejection?) The first step toward addressing these questions is identification of S allele products in the pollen and pistil. One strategy for identifying
The rationale for the loss-of-function failure of the S3 protein of P. inflata (5) was inserted in antisense orientation behind the promoter of the S3 gene, and the construct was introduced into the T-DNA region of a Ti plasmid, pBI101. The idea was to use the antisense S3 gene to inhibit the synthesis of S3 protein in transgenic plants and then examine whether this would abolish the ability of the transgenic plants to reject S3 pollen. The recombinant Ti plasmid was introduced into Agrobacterium tumefaciens, which was then used to infect strips of leaf tissue from P. inflata plants of S2S3 genotype. Upon infection, the T-DNA region of the Ti plasmid was transferred to the host genome; the integration of T-DNA occurs mainly by non-homologous recombination. To raise transgenic plants, appropriate tissue culture conditions (empirically determined) were used to allow the infected leaves to form shoots, then roots, and finally mature transgenic plants.

Six transgenic plants were found to have become completely self-compatible; they set large fruits when pollinated with self pollen. When total pistil protein from each of these transgenic plants was separated by cation-exchange chromatography (S proteins are among the few very basic proteins of the pistil), it was found that three of the transgenic plants produced a normal level of S2 protein but an undetectable level of S3 protein, and the other three produced very low levels of both S2 and S3 proteins. (Inhibition of S2 protein synthesis by the antisense S3 gene was most likely due to sequence similarity between the two S alleles.) Furthermore, the former three transgenic plants rejected S2 pollen but failed to reject S3 pollen, and the latter three transgenic plants failed to reject both S2 and S3 pollen. Thus, the correlation between the down-regulation of S2 and S3 proteins and the failure of the pistil to reject S2 and S3 pollen, respectively, strongly suggests
that S proteins are necessary for the pistil to recognize and reject self pollen.

For the gain-of-function approach, a genomic clone containing the S3 gene of *P. inflata* was introduced into *P. inflata* plants of S1S2 genotype by the same procedure described above. The rationale for this approach is that if S proteins alone are sufficient for the pistil to recognize and reject self pollen, then transgenic plants that produce the new S3 protein should acquire the ability to reject S3 pollen. Four transgenic plants were found to completely reject S3 pollen, indicating that they had acquired S3 allele specificity. These four transgenic plants all produced a normal amount of S2 protein from the S1 transgene, in addition to producing normal amounts of S1 and S2 proteins from the endogenous S1 and S2 alleles. Transgenic plants that failed to acquire the ability to reject S3 pollen did not produce any S3 protein. Thus, S proteins alone are sufficient to confer on the pistil the ability to reject self pollen. A similar conclusion was reached by McClure and coworkers (6) when using the gain-of-function approach to study an S protein of *Nicotiana alata*, another member of the Solanaceae.

The results from the loss-of-function and gain-of-function approaches provide direct evidence that S proteins control the pistil's ability to recognize and reject self pollen.

**Primary Structural Features of S Proteins**

The first cDNA for an S protein of a solanaceous species was isolated and sequenced by Clarke and coworkers (7). To date, cDNAs for more than 30 S proteins from 7 solanaceous species have been reported. All of the cDNA sequences predict the presence of a signal peptide, which is not found in a mature S protein; this is consistent with the finding by immunocytochemistry that S proteins are localized in the extracellular space of the transmitting tissue of the style, coinciding with the track of pollen tube growth (8). The sequences of S proteins are unusually divergent, with the amino acid sequence identity between different S proteins ranging from as low as 38% to as high as 98%. However, this sequence diversity is what one would expect for the products of S alleles (9). The reason is as follows. Any time a new S allele arises in a population, it is favored over existing alleles because its low frequency in the population ensures that pollen carrying the new S allele will be less likely to land on a stigma bearing the same S allele. So, one would expect selection pressure for sequence diversity to generate new S alleles.

A schematic representation of the primary structural features of S proteins is shown in Fig. 2. Two regions, designated HVa and HVb, exhibit the highest degree of sequence diversity (10). These two hypervariable regions are also the most hydrophilic regions of S proteins, raising the possibility that they may be involved in interactions with pollen S allele products and, thus, in determining S allele specificity. Despite the overall sequence diversity of S proteins, five regions are highly conserved; they are designated C1-C5 (10). The conserved regions C1, C4, and C5 contain mostly hydrophobic amino acids, and they are thought to be involved in the core structure of S proteins. The conserved regions C2 and C3 share a very high degree of sequence similarity with the corresponding regions of RNase T2 and a number of other RNases (11, 12). In fact, it was this sequence similarity that led to the discovery by Clarke and coworkers that S proteins are RNases (11). Among the amino acids conserved between S proteins and other RNases are the two histidines, one in the C2 region and the other in the C3 region. For RNase T2, these two histidines have been shown to be required for the RNase activity (13).

All of the S proteins that have been studied so far are glycoproteins with N-linked glycans. The number of potential N-glycosylation sites (with the consensus sequence asparagine-x-threonine-serine; x being any amino acid except proline) ranges from 1–5. Up to four N-linked glycan chains have been found with some S proteins (14). The N-glycosylation site near the C2 region is present in all except one S protein (Fig. 2).

**Involvement of RNase Activity of S Proteins in Pollen Rejection**

To determine whether the RNase activity of S proteins is an integral part of the S protein function in rejection of self pollen, a mutant S3 gene of *P. inflata* was engineered by replacing the codon for the conserved histidine in the C3 region, one of the two histidines implicated in RNase activity, with a codon for asparagine (15). This mutant S3 gene was introduced into *P. inflata* plants of S1S2 genotype by *Agrobacterium*-mediated transformation. Two transgenic plants were found to produce a normal amount of the mutant S3 protein. The mutant S3 protein indeed lacked RNase activity and, most importantly, the transgenic plants producing this mutant S3 protein failed to acquire the ability to reject S3 pollen. Thus, the RNase activity of S proteins is required for the rejection of self pollen. This finding, along with a previous finding by Clarke and coworkers that RNase of self pollen tubes, but not non-self pollen tubes, was degraded in the pistil (16), provides the answer to the question of how a pistil inhibits the growth of self pollen tubes. That is, the biochemical mechanism of self-rejection involves the cytotoxic action of the RNase activity of S proteins.

**S Allele Specificity Determinant Residing in Amino Acid Sequence of S Proteins**

To address the question of how an S protein only inhibits the growth of self pollen but not the growth of non-self pollen, requires identification of the S allele specificity determinant of S proteins and the pollen S allele products with which S proteins interact. The S allele specificity determinant is the region(s) of S proteins that distinguish(es) one S protein from other S proteins, and interact(s) with pollen S allele products. Since S proteins are glycoproteins, the S allele specificity determinant may reside in the carbohydrate moiety, the protein backbone, or both. To examine whether the carbohydrate side chains of S proteins are involved in S allele specificity, a mutant S3 gene of *P. inflata* was engineered by changing the codon for the only asparagine involved in N-glycosylation to a codon for aspartic acid (17). The mutant S3 gene was introduced into *P. inflata* plants of S1S2 genotype. Transgenic plants

---

Fig. 2. Schematic representation of the primary structure of S proteins. The five conserved regions are denoted C1–C5; the two hypervariable regions are denoted HVa and HVb. The location of the two histidines essential for RNase activity of S proteins and the location of the asparagine that is N-glycosylated in nearly all S proteins are indicated.
that produced a normal level of the non-glycosylated S\textsubscript{3} protein were found to reject S\textsubscript{3} pollen completely. Thus, the S allele specificity determinant of S proteins must reside in the amino acid sequence itself, and not in the glycan chains.

This result also suggests that nonglycosylated S proteins can be used for determining the three-dimensional structure by X-ray crystallography to circumvent the difficulty often associated with crystallizing glycoproteins. This difficulty can be expected in the case of S proteins, because the glycans attached to each site of S proteins has been found to be heterogeneous (14). Since up to a few micrograms of the nonglycosylated protein per pistil can be produced in transgenic plants, and it can be easily purified by a simple two-step procedure (4), it would be possible to use the transgenic plants to purify a large quantity of nonglycosylated S protein for X-ray crystallographical studies.

**Role of Hypervariable Regions of S Proteins in S Allele Specificity**

To examine the role of HVa and HVb regions in S allele specificity, two chimeric S genes of *P. inflata* were constructed using the S\textsubscript{1} and S\textsubscript{3} genes (S. Huang, A.G.M., and T.-h.K., unpublished results; see Fig. 3 for a schematic representation of the hybrid S proteins and summary of the results). The sequences of S\textsubscript{1} and S\textsubscript{3} proteins differ in 52 of 201 residues (5, 10): 34 of the differences are non-conservative replacements and 18 are conservative replacements. The differences are scattered throughout, but 12 of the 15 residues in the HVa region are different, and only one of these differences is a conservative replacement. In the HVb region, 4 of the 11 residues are different, and two of the differences are non-conservative replacements. Both chimeric S genes were constructed using the S\textsubscript{3} gene as the backbone. In one chimeric S gene, S\textsubscript{3}(HVa-HVb), both HVa and HVb regions of the S\textsubscript{3} gene and the region between them were replaced with the corresponding regions of the S\textsubscript{1} gene, and in the other, S\textsubscript{3}(HVb), only the HVb region of the S\textsubscript{3} gene was replaced with the corresponding region of the S\textsubscript{1} gene. Each chimeric S gene was introduced into *P. inflata* plants of S\textsubscript{S3} genotype to determine whether the transgenic plants acquired the ability to reject S\textsubscript{1} pollen, or S\textsubscript{3} pollen, or neither. Both hybrid S proteins produced in the transgenic plants were found to have comparable RNase activity as that of S\textsubscript{3} protein. However, both hybrid S proteins lost S\textsubscript{1} allele specificity and did not gain S\textsubscript{3} allele specificity, because the transgenic plants producing either hybrid S protein failed to acquire the ability to reject either S\textsubscript{1} or S\textsubscript{3} pollen. The transgenic plants did reject S\textsubscript{3} pollen because they produced endogenous S\textsubscript{3} protein. One interpretation of these results is that both hypervariable regions are necessary but not sufficient for S allele specificity. Thus, other regions of S proteins must also be required. Additional chimeric S genes will have to be examined to identify these other regions. These results also suggest that the RNase activity of S proteins is necessary but not sufficient for their function in self-incompatibility. That is, S proteins most likely contain two separate functional domains, an S allele specificity domain involved in recognition and an RNase activity domain involved in rejection. Thus, S proteins are recognition molecules with RNase activity, and they are different from other RNases, which share sequence similarity but are not involved in self-incompatibility (12, 18).

**Identity of the Pollen S Gene**

One key question in the study of self-incompatibility is: does the pistil S gene also control the self-incompatibility behavior of the pollen? The tripartite model put forward by Lewis predicts that the S locus is composed of three separate but closely linked parts: the S allele specificity part that determines the allelic specificity of both pollen and pistil, the pollen activity part, and the pistil activity part (19, 20). This hypothesis was based on the identification of pollen-part and pistil-part self-compatible mutants whose pollen behavior alone or pistil behavior alone in self-incompatibility interactions was affected. However, this hypothesis did not address the question of whether each part is controlled by a separate gene or not. Recently, Clarke and coworkers detected mRNA for S\textsubscript{2} and S\textsubscript{6} genes of *N. alata* in developing pollen, albeit at a level much lower than in the pistil (21). Sims and Clark (22) also detected mRNA for the S\textsubscript{1} gene of *Petunia hybrida* in developing anthers, but not in mature anthers, mature pollen, or *in vitro* germinated pollen tubes (22). Proteins that cross-react with antibodies specific to S\textsubscript{2} or S\textsubscript{6} protein of *N. alata* were also detected in hydrated pollen (21). The physiological relevance, if any, of these findings is not yet clear. Repeated attempts to identify S gene transcripts or S proteins in developing anthers or pollen of *P. inflata* have failed. Furthermore, the loss-of-function and gain-of-function experiments described earlier showed that expression of either the antisense S\textsubscript{3} gene or the new S\textsubscript{1} gene of *P. inflata* in the transgenic plants only altered the self-incompatibility behavior of the pistil, but not that of the pollen (4). In addition, up to 4 kb of the 5' upstream sequence of the *P. inflata* S\textsubscript{3} gene failed to express a reporter gene encoding β-glucuronidase in developing anthers or pollen of transgenic plants (H.-S. Lee and T.-h.K., unpublished results). These results suggest that it is likely that separate genes control pistil activity and pollen activity in self-incompatibility interactions. Hereafter, the discussion of the pollen S gene is based on the assumption that it is different from the pistil S gene. To date, the pollen S gene has not been identified.

**Predicted Characteristics of the Pollen S Gene**

Regardless of the molecular nature of the pollen S gene, it must be genetically linked to the pistil S gene; it must display a similar degree of allelic polymorphism as the pistil S gene; its allelic products must interact with S proteins; and its allelic products must be as divergent in sequence as S proteins in order for them to distinguish between different S proteins.

<table>
<thead>
<tr>
<th>RNase Activity</th>
<th>S3 allele specificity</th>
<th>S1 allele specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3(HVa-HVb)</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>S3(HVb)</td>
<td>Normal</td>
<td>No</td>
</tr>
</tbody>
</table>

Fig. 3. Schematic representation of two hybrid S proteins between S\textsubscript{1} and S\textsubscript{3} proteins, and a summary of their properties. S3(HVa-HVb) contains most of the S\textsubscript{3} protein except that the HVa and HVb regions, and the region between them have been replaced with the corresponding regions of the S\textsubscript{1} protein. S3(HVb) contains most of the S\textsubscript{3} protein except that the HVb region has been replaced with the corresponding region of the S\textsubscript{1} protein. The RNase activities of both hybrid S proteins are comparable to the RNase activity of the wild-type S\textsubscript{3} protein.
Despite the predicted genetic linkage between the pollen S gene and the pistil S gene, the approach of chromosome walking to identify the pollen S gene is most likely not feasible for the following reasons. First, there is evidence that recombination is suppressed at the S locus (9); the physical distance between the pollen S gene and the pistil S gene may be much greater than the genetic distance. Second, perhaps due to the lack of recombination, the S locus has accumulated a substantial number of repetitive sequences (23). In fact, repetitive sequences are found immediately upstream and downstream of the transcribed region of the S gene, and there is no sequence similarity between the corresponding flanking regions of different S alleles (23). Ongoing efforts to identify the pollen S gene are thus focusing on strategies based on the other characteristics of the pollen S gene.

**Models for Allele-Specific Inhibition of Pollen**

Because a pistil distinguishes between self pollen and non-self pollen based on whether or not the S allele carried by the pollen matches either one of the S alleles carried by the pistil, S proteins must interact with pollen S allele products to elicit such allele-specific inhibition. Two possible ways by which allele-specific inhibition can be accomplished (24, 25) are shown in Fig. 4.

First, the S allele-specific inhibition may lie in the allele-specific uptake of S proteins into pollen tubes (Fig. 4A). This model predicts that the pollen S gene product is a receptor or gatekeeper, which allows S proteins to enter pollen tubes in an S allele-specific manner. As illustrated in Fig. 4A, an S1 pollen tube is growing in a pistil carrying S1 and S2 alleles. The transmitting tissue of the style produces and secretes S1 and S2 proteins into the extracellular space where the S1 pollen tube is growing. The growth of the S1 pollen tube would be inhibited because this pollen tube produces a receptor that allows S1 protein to enter. Once inside, the RNase activity of S1 protein would degrade pollen tube RNA (rRNA and/or mRNA) leading to the inhibition of tube growth. If an S3 pollen tube is growing in the same style, it would not be inhibited, because the receptor produced by the S3 pollen would not allow either S1 or S2 protein to enter.

Alternatively, the S allele-specific inhibition may lie in the allele-specific inhibition of RNase activity of S proteins (Fig. 4B). This model predicts that the pollen S gene product is an RNase inhibitor, which resides inside pollen tubes. This inhibitor obstructs the RNase activity of all non-self S proteins, but does not inhibit the RNase activity of self S protein. As illustrated in Fig. 4B, an S1 pollen tube is growing in a pistil carrying S1 and S2 alleles. According to this model, uptake of S proteins is not allele specific, so both S1 and S2 proteins would be able to enter the S1 pollen tube. However, only self S protein, in this case S1 protein, would be able to function inside the S1 pollen tube, because the RNase inhibitor produced by the S1 pollen tube would inhibit the RNase activity of S2 protein, but not that of S1 protein. Thus, the growth of the S1 pollen tube would be inhibited. If an S3 pollen tube is growing in the same style it would escape inhibition because the RNase inhibitor produced by this pollen tube would inhibit the RNase activity of both S1 and S2 proteins. One way this allele-specific inhibition of S proteins could be accomplished is also depicted in Fig. 4B. Both S1 and S2 proteins are shown to contain two separate functional domains, an S allele specificity domain and an RNase activity domain. In the case of S1 protein, the RNase inhibitor produced by the S1 pollen would interact with the allele-specific domain of S1 protein through the matching of the S allele specificity domain, and in so doing the RNase

**Fig. 4.** Two models for allele-specific inhibition of pollen. (A) Receptor or gatekeeper model. (B) RNase inhibitor model. Each model depicts an S1 pollen tube growing in the extracellular space of the transmitting tissue of a style that produces S1 and S2 proteins. In A, only self S protein, S1 protein, is able to enter the S1 pollen tube, whereas in B, both self (S1) and non-self (S2) S proteins are able to enter the S1 pollen tube, but only S1 protein is able to function as an RNase. In both models, the RNase activity of S1 protein is responsible for growth inhibition of the S1 pollen tube.
activity domain of S1 protein would not be affected. In the case of S2 protein, the RNase inhibitor produced by the S1 pollen would interact with the RNase activity domain, instead of the S allele-specific domain, of S2 protein, and in so doing would inhibit the RNase activity of S2 protein.

There is genetic evidence supporting the RNase inhibitor model. It has been shown that self-incompatibility breaks down when a diploid self-incompatible plant is converted to a tetraploid plant through tissue culture manipulations, and that this breakdown is caused by the inability of the pollen carrying two different S alleles to be rejected by the pistil (reviewed in refs. 2 and 3). For example, a tetraploid plant of S1S1S2S2 genotype produces three genotypes of diploid pollen: S1S1, S1S2, and S2S2; both S1S1 and S2S2 pollen are rejected by the pistil, but the S1S2 pollen fails to be rejected by the pistil. These results can be explained by the RNase inhibitor model in that the RNase activity of both S1 and S2 proteins taken up by the S1S2 pollen tubes would be inhibited by the RNase inhibitor produced by S2 and S1 alleles of pollen, respectively, thus leading to the inability of the pistil to reject the S1S2 pollen.

However, the results from a recent study of the effect of a mutant S3 protein lacking RNase activity on the function of wild-type S1 protein seem to support the receptor model (A.G.M., Y. Chung, and T.-h.K., unpublished results). The codon for the conserved histidine in the C3 region of the S1 protein was replaced with a codon for arginine; this mutant S1 gene was introduced into transgenic plants of S2S3 genotype. It was found that the mutant S1 protein had a dominant negative effect on the wild-type S1 protein, but not on the wild-type S2 protein. That is, transgenic plants producing wild-type S2 and S3 proteins, as well as the mutant S1 protein, failed to completely reject S1 pollen, but rejected S2 pollen completely. One possible explanation for these results is that the mutant S1 protein competes with the wild-type S1 protein for binding to the receptor produced by the pollen S1 allele, and because the mutant S1 protein lacking RNase activity cannot inhibit S1 pollen, some of the S1 pollen escape inhibition. This interpretation is consistent with the conclusion stated earlier that S proteins have two separate functional domains and thus a mutation abolishing the RNase activity does not affect the function of S allele specificity domain in interacting with the pollen S allele products. An important implication of these results is that such an RNase(-) form of S protein may be expressed in yeast for use in the two-hybrid protein–protein interaction assay to identify the pollen S gene, because wild-type S proteins might be expected to be cytotoxic to yeast cells.

Certainly, the results presented above can also be explained by other models, and it is not until the pollen S gene has been identified that one can assess the validity of these two or any other alternative models.

**Evolutionary Aspects of Self-Incompatibility**

Among the families that display gametophytic self-incompatibility, the Solanaceae has been studied most extensively at the molecular level. However, recently, cDNAs encoding S proteins have been identified and sequenced from species of three other families: *Malus × domestica* (apple) and *Pyrus serotina* (Japanese pear) of the Rosaceae (26, 27), *Antirrhinum hispanicum* of the Scrophulariaceae (28), and *Papaver rhoas* of the Papaveraceae (29). The S proteins of the species in the former two families are similar to the S proteins of solanaceous species, but the S proteins of the species in the latter family show no similarity to the S proteins of solanaceous species, or to any other proteins in the data bases. Based on the molecular nature of the S proteins, it would seem reasonable to infer that the Rosaceae and Scrophulariaceae employ the same mechanism as the Solanaceae in self-incompatibility interactions, whereas the Papaveraceae employs a different mechanism. Thus, gametophytic self-incompatibility is not monophyletic.

Phylogenetic studies of the S proteins from different solanaceous species have revealed that certain S proteins of one species are more closely related to some S proteins of other species than to other S proteins of the same species (30). These results have been interpreted to mean that S allele polymorphism predates speciation in the Solanaceae. That is, gametophytic self-incompatibility displayed by the extant solanaceous species existed in their common ancestor. A similar conclusion has been made regarding gametophytic self-incompatibility displayed by the extant rosaceous species based on phylogenetic studies of the S protein sequences from two rosaceous species (27). However, this RNase-based gametophytic self-incompatibility system appears to have arisen multiple times over the course of evolution, because phylogenetic studies of the sequences of S proteins from the Solanaceae, Rosaceae, and Scrophulariaceae have revealed that S proteins of each family are more closely related to each other than to S proteins of other families (27, 28).

As for the origin of the RNase-based self-incompatibility system, it is tempting to speculate that the system may have evolved by recruiting RNases that were involved in defense against pathogens for use in the rejection of self pollen tubes. In fact, a number of extracellular RNases that show sequence similarity with S proteins have been identified in pistils and other tissues of both self-incompatible and self-compatible species (12). Although the functions of these RNases, termed S-like RNases, have not been determined, defense against pathogen infection is a possible function. The pistil is rich in extracellular nutrients because it has to support the growth of pollen tubes, so it would be expected to be particularly vulnerable to pathogen infection. However, very few pathogens are known to infect plants through the pistil. A likely reason for this is that the presence of a battery of pathogenesis related proteins, including chitinases and glucanases (ref. 31; A.G.M., unpublished results), and RNases may play a role in protecting the pistil from pathogen infection. Interestingly, the pistil of *P. inflata* has been found to be particularly rich in RNases (32), one of which, RNase X2, is an extracellular RNase and shares a very high degree of sequence similarity with S proteins even though it is not involved in self-incompatibility (18).

**Practical Applications of Self-Incompatibility**

One application of self-incompatibility is in hybrid seed production. Hybrid seed is obtained from pollination between two plants with different genetic backgrounds. In many species the phenomenon of heterosis or hybrid vigor results after such a cross and the progeny shows increased vigor and a concomitant higher yield. However, since most cultivated crop species are self-compatible (because breeders selected out the self-incompatibility trait to generate inbred lines), hybrid seed production requires manual or mechanical removal of anthers from the plant serving as female parent to prevent self-fertilization; this is a very labor-intensive and costly process. If self-incompatibility can be restored in self-compatible crop species, it would facilitate hybrid seed production without emasculation. One way to do this is by transforming crop species with the pollen and pistil S genes, once the pollen S gene has been identified.

For some species, self-incompatibility is actually a nuisance rather than a benefit. For example, most fruit trees are self-incompatible; so to produce fruits, farmers usually have to plant two different varieties in adjacent rows, using one variety as nothing but a pollen donor. This obviously results in waste in land use and problems in managing different cultivars. It would be beneficial to farmers if self-incompatibility could be broken down in fruit tree species; this is certainly feasible.
Conclusions

Gametophytic self-incompatibility displayed by members of the Solanaceae, Rosaceae, and Scrophulariaceae families uses S proteins, a unique class of RNases, as both recognition molecules and cytotoxic agents to specifically destroy self pollen to prevent inbreeding. Although it is now clear that the rejection of self pollen is mediated by the RNase activity of S proteins, it is still not known how an S protein distinguishes between self and non-self pollen. The answer to this question will require the identification of the so far elusive male component, the pollen S gene and of the region(s) of S proteins involved in the interactions with the pollen S allele products. Elucidation of this RNase-based self/non-self-recognition mechanism will expand the scope of our understanding of the biological roles of RNases. As to the practical aspects, it is hoped that through the study of this mechanism, we will be able to manipulate the breeding behavior of agronomically important species to the benefit of breeders and farmers.

The work carried out in our laboratory was supported by Grant IBN 92-20145 from the National Science Foundation.