The *S*–locus and unilateral incompatibility

C. Nathan Hancock, Katsuhiko Kondo, Brian Beecher and Bruce McClure

References	**Article cited in:** http://rstb.royalsocietypublishing.org/content/358/1434/1133#related-urls
Rapid response	Respond to this article http://rstb.royalsocietypublishing.org/letters/submit/royptb;358/1434/1133
Email alerting service	Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. B* go to: **http://rstb.royalsocietypublishing.org/subscriptions**

The *S*-locus and unilateral incompatibility

C. Nathan Hancock, Katsuhiko Kondo, Brian Beecher† and Bruce McClure[*]

Department of Biochemistry, University of Missouri-Columbia, 117 Schweitzer Hall, Columbia, MO 65211, USA

Plants have many ways to regulate the type of pollen that arrives on the stigma surface. Once there, further control mechanisms regulate compatibility. The latter controls are largely based on biochemical interactions that support compatible pollination and prevent incompatible matings. S-RNase-based self-incompatibility (SI) systems are the most phylogenetically widespread mechanisms for controlling pollination. Studies of *Nicotiana* establish a firm link between SI and unilateral interspecific incompatibility. Although implicated in both inter- and intraspecific compatibility, S-RNase operates through at least three distinct genetic mechanisms that differ in their dependence on non-S-RNase factors. Identification and characterization of these non-S-RNase factors is currently an area of active research. Searching for genetic and biochemical interactions with S-RNase can identify candidate non-S-RNase factors. HT-protein is one factor that is required for *S*-allele-specific pollen rejection in the Solanaceae. Major style arabinogalactan proteins such as TTS interact biochemically with S-RNase. These glycoproteins are known to interact with compatible pollen tubes and have long been suggested as possible recognition molecules. Their binding to S-RNase implies a link between stylar systems for compatibility and incompatibility. Thus, genetic and biochemical studies suggest a highly networked picture of pollen–pistil interactions.

Keywords: *Nicotiana*; pollination; self-incompatibility; interspecific incompatibility; unilateral incompatibility

1. INTRODUCTION

In flowering plants the pistil serves to catch pollen, provide an environment for it to germinate and then guide the pollen tube to the ovule where fertilization takes place. Plants have many strategies to control the type of pollen that arrives on the stigma. A flower's colour or the time of day when it is open can affect interactions with pollinators and, hence, the type of pollen received. Once pollen arrives on the stigma, however, biochemical interactions are the main avenues for controlling fertilization. These interactions are, therefore, crucial to a species' success. There is a need to discriminate between undesirable pollen and desirable pollen that is likely to generate successful progeny. Then, there is a need to inhibit the growth of undesirable pollen or support the growth of desirable pollen. The pollen–pistil interactions that address these needs allow plants to control their mating and, hence, their evolutionary success.

It is useful to consider the window of genetic relatedness for optimum mating. For plants that are susceptible to inbreeding depression, it is important to avoid selfing and crosses between very closely related individuals. At the other end of the spectrum, it is also necessary to avoid crosses that are too wide. Interspecific crosses can lead to inviable zygotes or sterile offspring. Thus, the functions of the pistil are to recognize and reject pollen from plants that are too closely related, support the growth of pollen from more distantly related plants of the same species, and reject pollen from other species. All these functions are facets of the overall function of the pistil, which is to control mating.

The interactions that control mating take place in the extracellular matrix of the pistil. In *Nicotiana*, pollen germination takes place near an oil–aqueous interface in the secretory zone of the stigma (Lush *et al.* 1998). Pollen tubes grow towards the ovary through the matrix secreted in the transmitting tract (Cheung 1996). Thus, these secretions are likely to contain the factors necessary for supporting compatible pollen and for recognizing and rejecting incompatible pollen.

There are two main paradigms for understanding the action of these factors. The incongruity paradigm focuses on coadaptation between the pollen and the pistil (Hogenboom 1984). There is clearly intense selection for a productive interaction between partners. Compatibility occurs when multiple pistil factors interact productively with multiple pollen factors. Pollination fails, or is incongruous, when these factors are not well matched (Hogenboom 1984). This paradigm is useful for understanding failure of pollinations between unrelated species. Incompatibility is an alternative paradigm that describes the failure of pollinations as the result of an active process that interferes with an otherwise compatible cross (de Nettancourt 1997, 2001). The critical distinction is that incongruity is regarded as a passive process and incompatibility is regarded as active rejection. Incompatibility is clearly the more useful paradigm for pollen rejec-

[*]Author for correspondence (mcclureb@missouri.edu).

† Present address: Department of Agronomy and Horticulture, University of Nebraska-Lincoln, PO Box 830915, Lincoln, NE 68583-7904, USA.

Phil. Trans. R. Soc. Lond. B (2003) **358**, 1133–1140
DOI 10.1098/rstb.2003.1284

1133

tion within species. As the genetic distance between mating partners increases either incompatibility or incongruity (or both) may operate.

This review describes the relationship between SI and UI in *Nicotiana*. Both SI and UI rely on ribonuclease-based pollen rejection. Ribonuclease-based pollen rejection occurs in diverse plants and the mechanism is one of the better understood pollen–pistil interactions. Thus, research on specific ribonuclease-based systems may contribute to a general understanding of compatibility in many flowering plants. By far, the most progress has been made towards understanding ribonuclease-based SI. However, insights from SI will inform studies of ribonuclease-based UI. In addition, as more is learned about these incompatibility systems, it is likely we can gain insight into the incongruity mechanisms that control even wider crosses.

2. S-RNase

SI systems are the best-studied mechanisms for controlling pollination. The most phylogenetically widespread SI system uses ribonucleases expressed in the pistil to recognize and reject self-pollen or pollen from closely related plants. The specificity of pollen rejection is controlled by a multiallelic *S*-locus (de Nettancourt 1997, 2001). SI species in the Solanaceae, Rosaceae and Scrophulariaceae have ribonuclease-based systems (Igic & Kohn 2001). The system is classified as gametophytic SI because pollen is rejected when its single *S*-allele is the same as one of the *S*-alleles in the diploid pistil (de Nettancourt 2001). The *S*-locus encodes at least two different genes that, by definition, determine the specificity of pollen rejection. S-RNase is the product of the *S*-locus that is expressed in the pistil. Lai *et al.* (2002) and others have identified an excellent candidate that fulfils important criteria expected of pollen S (Dowd *et al.* 2000). However, at this point, the results are not definitive, and this review focuses on the activities of the style in pollen rejection.

S-RNases were originally identified simply as abundant stylar proteins that co-segregated with a pollen rejection phenotype (Bredemeijer & Blaas 1981; Anderson *et al.* 1986). Bredemeijer & Blaas (1981) used isoelectric focusing of style extracts from *Nicotiana alata* to show that plants that rejected S_2 pollen always displayed a unique protein. Similar results were obtained for other genotypes. The *S*-allele-specific proteins focused in the basic region of the gel and accumulated late in the pistil development. Anderson *et al.* (1986) sequenced the N-terminus of the S_2 glycoprotein and cloned the cDNA. Similar *S*-glycoproteins were subsequently cloned from many other species, genera and families (Igic & Kohn 2001). The proteins showed many characteristics expected of recognition proteins involved in pollen rejection. They were expressed in the stigma, style and ovary, and accumulated to high levels in the extracellular matrix where they could interact with pollen tubes. Sequence analysis showed short conserved regions flanked by extensive variable regions (Ioerger *et al.* 1991). Kawata *et al.* (1988) noted that two of the conserved regions were similar to active site regions of RNaseT$_2$ from *Aspergillus oryzae*. McClure *et al.* (1989) tested five *S*-glycoproteins from *N. alata* and showed that

each possessed intrinsic ribonuclease activity. These proteins are now known as S-RNases.

The cytotoxic model is now widely accepted as the basis for S-RNase-based pollen rejection. In this model, S-RNases function as both the specificity determinants in the pistil and as cytotoxins that directly inhibit growth of undesirable pollen.

The most straightforward predictions from this model are that pollen rejection is associated with a cytotoxic action against pollen and that ribonuclease activity is required for pollen rejection. Both these predictions have been experimentally confirmed. McClure *et al.* (1990) labelled pollen RNA *in vivo* by growing plants in the presence of [^{32}P]orthophosphate. The radioactive pollen was used in controlled pollinations, RNA was isolated at various times post-pollination and detected by autoradiography. Pollen RNA recovered from compatible pollinations appeared normal. However, pollen RNA from incompatible pollinations was degraded. This was interpreted as direct evidence that S-RNases act as *S*-allele-specific cytotoxins (McClure *et al.* 1990; Gray *et al.* 1991). Huang *et al.* (1994) showed that ribonuclease activity is required for pollen rejection. Using site-directed mutagenesis, they converted one of the histidine residues required for enzymatic activity of S_3-RNase from *Petunia inflata* to either asparagine or arginine. The inactive S-RNases were not able to cause the rejection of S_3 pollen even when expressed at high levels (Huang *et al.* 1994).

Together, these results strongly support the major tenets of the cytotoxic model. However, Lush & Clarke (1997) reported that pollen tubes can recover from inhibition by S-RNase, suggesting that the cytotoxicity is reversible. In addition, an electron microscopy study of ribosome and polysome distribution was interpreted as being inconsistent with a straightforward cytotoxic model (Walles & Han 1998; de Nettancourt 2001). Thus, although most evidence supports the cytotoxic model, further discoveries may require its revision.

3. S-RNase AND INTERSPECIFIC UI

Far less is known about interspecific pollination barriers than about intraspecific barriers. One reason for this is the difficulty of performing genetic analyses of interspecific systems. Normal chromosome segregation, recombination and fertility cannot be assumed in interspecific crosses. Also, there are likely to be many different reasons why interspecific pollinations fail. The stigma may be challenged with pollen from many different species and there is no reason to expect a single rejection mechanism will protect against all of them. As the genetic distance between species increases, the failure of pollination is likely to be better explained by incongruity rather than incompatibility.

UI systems have much to offer in studies of interspecific pollen rejection. Pollination failure is unlikely to be due to gross differences in the requirements for pollen tube growth because crosses are compatible in one direction. In addition, there are instances where UI closely parallels SI. Here, knowledge of SI systems can provide insight into the control of pollen flow between species.

The parallel between SI and UI was clearly described by Lewis & Crowe (1958). They described several UI sys-

tems that follow the SI × SC rule whereby pollen from the SI species is compatible on the SC pistil, but the reciprocal cross is incompatible. They noted some cases where pollen from SC species was compatible on SI pistils, but these involved SC species where SI races exist. The SI × SC rule applied to families that display gametophytic SI and families with sporophytic SI. Although it is not absolute, the consistency of the SI × SC rule suggests a link between inter- and intraspecific pollen rejection. The suggested linkage is that the *S*-locus controls UI as well as SI. The pistil of the SI species (i.e. the partner with a fully functional *S*-locus) typically rejects pollen from SC species as well as its own pollen. Thus, the *S*-locus fulfils the function alluded to above; it defines a window of genetic relatedness for compatible pollinations.

Genetic studies support a role of the *S*-locus in both SI and UI. *Lycopersicon* offers advantages for such studies because it contains SI and SC species with the same chromosome number. The *S*-locus has been implicated in UI between SC *L. esculentum* and SI *L. hirsutum* (Martin 1967; Bernacchi & Tanksley 1997) as well as *L. pennellii* (Chetelat & DeVerna 1991). Pandey (1973) showed that *S*-alleles behave differently in interspecific crosses in *Nicotiana*. Four *S*-alleles from *N. bonariensis* were tested for interspecific compatibility. The *S*-alleles behaved differently; for example, S_1 and S_2 caused the rejection of SC *N. glauca* pollen but S_2 and S_3 did not. Different patterns were observed with pollen from different species (Pandey 1973). The differential response to different *S*-alleles directly implicates the *S*-locus in interspecific pollen rejection. Hiscock & Dickinson (1993) found that most of the crosses between SI and SC species in the Brassicaceae also follow the SI × SC rule. They also showed that treatments such as bud pollination, commonly used to overcome SI, interfere with UI (Hiscock & Dickinson 1993). It is noteworthy that SI in *Brassica* is totally different from S-RNase-based SI (de Nettancourt 2001) and yet the SI × SC rule still has predictive value. Thus, there is strong genetic evidence that the *S*-locus is involved in some UI systems.

4. MULTIPLE MECHANISMS FOR S-RNase-DEPENDENT UI IN *NICOTIANA*

Plant transformation circumvents some of the difficulties that are inherent in UI studies. Murfett *et al.* (1994) reported an efficient expression system for S-RNase. Using a tomato chitinase promoter and the downstream region of the *N. alata* S_{A2}-RNase gene, they reported that a high proportion of transformed plants gained the ability to reject S_{A2} pollen. S_{A2}-RNase was active, correctly processed and accumulated to levels comparable to expression from the native promoter in SI *N. alata* $S_{A2}S_{A2}$. The ability of a single S-RNase gene to confer *S*-allele-specific pollen rejection convincingly showed that S-RNase contains all the information necessary for intraspecific pollen recognition. The same expression system has been used to test hypotheses about the role of S-RNase in UI. This approach allows the effect of a single gene, S-RNase, to be determined without complications arising from other factors.

The analysis of the UI between SI *N. alata* and SC *Nicotiana* species revealed the potential complexity of

interspecific UI (Murfett *et al.* 1996). Most accessions of *N. alata* are SI. However, some horticultural accessions are SC. The SC cultivar Breakthrough, which does not express S-RNase, has proven useful for studies of the role of S-RNase and other factors in pollen rejection. SC *N. tabacum* and SC *N. glutinosa* are examples of species that do not follow the SI × SC rule. Pollen from these species is rejected by both SI and SC accessions of *N. alata*. However, pollen from SC *N. plumbaginifolia* is only rejected by SI *N. alata* as predicted by the SI × SC rule. *N. tabacum* and *N. plumbaginifolia* were transformed with S_{A2}- or S_{C10}-RNase from *N. alata* to test whether S-RNase has a role in these UI systems. The results of these experiments were complex and showed three distinct UI mechanisms (i.e. an S-RNase independent mechanism and two S-RNase dependent mechanisms (Murfett *et al.* 1996)). Together with the intraspecific SI mechanism there are four different pollen rejection mechanisms operating even in this small group of species. If this is representative of the mechanistic diversity of interspecific pollen rejection the potential for complexity is staggering.

The mechanisms for rejecting *N. tabacum* and *N. glutinosa* pollen are the simplest. Because the SC cultivar of *N. alata* retains the ability to reject their pollen, it is clear that an S-RNase-independent mechanism contributes (Murfett *et al.* 1996). However, transgenic *N. tabacum* and *N. plumbaginifolia* plants expressing either S_{A2}- or S_{C10}-RNase gained the ability to reject pollen from these two species. Thus, *N. tabacum* and *N. glutinosa* are also sensitive to S-RNase-dependent pollen rejection. Two totally different mechanisms contribute to UI between these SC species and SI *N. alata*. This is an important lesson. It cannot be assumed that pollination failure is caused by a single mechanism. It is also noteworthy that the S-RNase-dependent pathway does not depend on other factors from *N. alata*. This type of pollen rejection is, therefore, referred to as 'factor independent' (McClure *et al.* 2000). As research into this mechanism has progressed it is apparent that it may be more accurate to state that factors expressed in *N. tabacum* and *N. plumbaginifolia* can substitute for *N. alata* factors in S-RNase-dependent rejection of pollen from *N. tabacum* and *N. glutinosa*.

The rejection of *N. plumbaginifolia* pollen is more complex (Murfett *et al.* 1996). As the SC cultivar of *N. alata* accepts *N. plumbaginifolia* pollen, it is clear that the S-RNase-independent mechanism does not contribute to this type of UI. Transgenic *N. tabacum* and *N. plumbaginifolia* plants expressing S-RNase also remain compatible with *N. plumbaginifolia* pollen. Thus, S-RNase alone is not sufficient to cause *N. plumbaginifolia* pollen rejection. However, when transgenic *N. plumbaginifolia* plants are crossed with SC *N. alata* the resulting hybrids did reject *N. plumbaginifolia* pollen. These transgenic hybrids also reject pollen from SI *N. alata*; whereas, the primary transformants did not. Furthermore, antisense experiments showed that when S-RNase expression was suppressed, *S*-allele-specific pollen rejection and the ability to reject *N. plumbaginifolia* pollen were always affected in parallel (Murfett *et al.* 1996).

There are clearly several distinct pathways for S-RNase-dependent pollen rejection in *N. alata* (McClure *et al.* 2000). Pollen from SC *N. tabacum* and *N. glutinosa* is sensitive to 'factor-independent' rejection. The *N. alata* ×

N. plumbaginifolia UI system requires both S-RNase and other non-S-RNase factors. The intraspecific SI system shows a similar requirement for both S-RNase and non-S-RNase factors. The latter mechanisms are referred to as 'factor dependent' to emphasize their dependence on non-S-RNase factors from *N. alata*. The nature of the 'factors' is currently an area of active research. It is likely that several factors are required.

Factor-dependent SI and UI are distinguished by their specificity (McClure *et al.* 2000). SI is *S*-allele-specific; a single S-RNase causes rejection of a single pollen S genotype. UI with *N. plumbaginifolia* is less specific. With one exception every *N. alata* *S*-allele tested causes rejection of *N. plumbaginifolia* pollen. S_{9811}-RNase functions normally in SI but does not cause rejection of *N. plumbaginifolia* pollen (Beecher & McClure 2001).

Together, the transgenic plant studies demonstrate a definite connection between SI and UI. Transforming plants with a single cloned S-RNase gene can cause rejection of pollen from SC species. The interactions between the S-RNase transgene and the genetic background show that there are at least three different S-RNase-dependent pollen rejection mechanisms. In addition, an S-RNase-independent mechanism contributes to UI between *N. alata* and *N. tabacum* and *N. glutinosa*. This conclusion has important implications for understanding interspecific pollen rejection.

One implication is that products of the *S*-locus function in multiple genetic pathways. Discrimination between inter- and intraspecific pollen may be regarded as separate functions. S-RNase is clearly capable of functioning efficiently in pollen rejection, and its recruitment to fulfil both functions may be an example of parsimonious use of the genetic tool kit. Perhaps it is not surprising that it requires different non-S-RNase factors to function against pollen from different species. However, insofar as inter- and intraspecific pollen rejection mechanisms share factors (i.e. S-RNase and possibly other factors), they are networked.

Another implication is that pollen from a single species may be susceptible to more than one rejection mechanism. For instance, *N. tabacum* pollen is susceptible to S-RNase-dependent and S-RNase-independent rejection. Redundant pollen rejection mechanisms probably explain many exceptions to the SI × SC rule. For example, *N. alata* cv. Breakthrough is SC because it does not express S-RNase. However, it retains UI with *N. tabacum* because it retains the S-RNase-independent rejection mechanism. The existence of multiple, sometimes redundant, pollen rejection mechanisms operating between species explains the difficulty of genetic approaches to understanding interspecific pollen rejection.

5. S-RNase HAS A UNIQUE ROLE IN INTERSPECIFIC POLLEN REJECTION

The relative lack of *S*-allele-specificity in UI prompts the question of whether S-RNase is uniquely adapted for pollen rejection or whether it can be replaced by another RNase. The ability to test the effects of recombinant RNase expression on pollination allows this question to be explored in detail.

Beecher *et al.* (1998) tested whether a non-S-RNase could substitute for S_{A2}-RNase in *N. plumbaginifolia* pollen rejection. RNaseI from *Escherichia coli* was chosen because it is an extracellular enzyme in the same family as S-RNases and has similar charge and size characteristics (Beecher *et al.* 1998). The *E. coli* RNaseI gene was engineered for expression in plants by replacing the bacterial secretion signal with the S_{A2}-RNase signal peptide. An intron was engineered into the gene, and S_{A2}-RNase gene 3′ untranslated regions were inserted downstream of the coding sequence. Style-specific expression was obtained with the tomato chitinase gene promoter. Constructs were initially transformed into *N. plumbaginifolia*, screened for expression and crossed with SC *N. alata*.

Second generation transformed hybrids were tested for the expression of RNaseI and the pollination phenotype. The bacterial gene was expressed only in the mature pistil. The enzyme was active and accumulated in the extracellular matrix as expected. As the expression level is a critical parameter, quantitative immunoblot analysis was performed to ensure that hybrids were expressing amounts of RNaseI that were comparable to the levels of S_{A2}-RNase expressed in controls. Six hybrids expressing RNaseI at between 19 and 33 μg mg^{-1} of style protein were compared with six hybrids expressing S_{A2}-RNase at levels ranging from 19 to 29 μg mg^{-1}. As *E. coli* RNaseI has a higher specific activity than S_{A2}-RNase, the ribonuclease activities in the hybrids expressing RNaseI were much higher than in the S_{A2}-RNase hybrids (i.e. 120 to 226 A$_{260}$ U min^{-1} mg^{-1} in the RNaseI hybrids versus 29 to 54 A$_{260}$ U min^{-1} mg^{-1} in the S_{A2}-RNase hybrids).

Style squashes were used to assess compatibility. All six control hybrids expressing S_{A2}-RNase rejected *N. plumbaginifolia* pollen, none of the hybrids expressing RNaseI showed any rejection. Hybrids expressing S_{A2}-RNase accepted *N. alata* S_{C10} pollen (28 compatible in 30 pollinations), so rejection of *N. plumbaginifolia* pollen (0 compatible in 30 pollinations) could not be attributed to other defects. The RNaseI hybrids were fully compatible with both S_{C10} pollen and *N. plumbaginifolia* pollen (i.e. 30 compatible in 30 pollinations).

These results add to our understanding of the role of S-RNase in UI. As discussed in §4, UI between *N. plumbaginifolia* and *N. alata* differs from SI in that the latter shows a much higher degree of specificity. However, a non-S-RNase could not substitute for S-RNase in UI, suggesting that S-RNases are uniquely adapted for pollen rejection.

This hypothesis has been tested more extensively in the factor-independent system for *N. tabacum* pollen rejection (Beecher 2001). The effects of four RNases on *N. tabacum* pollen tube growth were compared. Three S-RNases with a range of properties were tested: S_{C10}-RNase, S_{9811}-RNase and S_{Con5}-RNase. S_{C10}-RNase from *N. alata* was used as a positive control. Its behaviour is typical of most S-RNases in that it is active in the intraspecific *S*-allele-specific mechanism and in both factor-dependent and factor-independent interspecific pollen rejection. S_{9811}-RNase functions normally in SI but does not cause factor-dependent rejection of *N. plumbaginifolia* pollen. S_{Con5}-RNase is a chimera composed of sequences from S_{A2}-RNase and S_{C10}-RNase (Zurek *et al.* 1997; Beecher & McClure 2001). It is an active ribonuclease and is
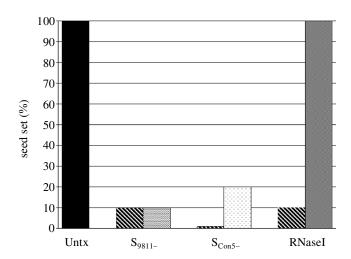
Figure 1. The seed set in *N. tabacum* expressing recombinant RNases. Each plant was pollinated 10 times with pollen from untransformed *N. tabacum*. Data are shown for a plant expressing S_{9811}-RNase, S_{Con5}-RNase or RNaseI from *E. coli*. Each of these plants is paired with a plant expressing S_{C10}-RNase (left, hatched bars) at a comparable level. Abbreviation: Untx, untransformed *N. tabacum* expressing no recombinant RNase.

expressed normally in transgenic plants, but it does not cause *S*-allele-specific pollen rejection. *E. coli* RNaseI was used as a non-S-RNase. All four RNases were engineered for expression and constructs were transformed into *N. tabacum.*

All four RNases were expressed normally and were active enzymes, but only the S-RNases caused pollen rejection. Quantitative immunoblot analyses were used to select plants expressing comparable amounts of recombinant protein, and the pollination phenotypes were monitored by both style squashes and seed set. Figure 1 shows a generalized comparison of the pollen rejection abilities of the four RNases. The figure presents seed-set data for plants expressing S_{9811}-RNase, S_{Con5}-RNase or RNaseI compared with a plant expressing a comparable amount of S_{C10}-RNase (i.e. the positive control). The general comparison between the S-RNases and the non-S-RNase is clear. Although their pollen rejection abilities vary widely, the S-RNases were all capable of causing rejection of *N. tabacum* pollen. By stark contrast, plants expressing *E. coli* RNaseI behaved exactly as untransformed controls, and full seed set was observed in every cross.

The overall conclusion from these experiments is that S-RNases possess some special feature that allows them to function in pollen rejection. Clearly, ribonuclease activity *per se* is not sufficient for any of the RNase-dependent mechanisms. This is obvious in the *S*-allele-specific SI system where pollen tubes are susceptible to only one of the two S-RNases expressed in a diploid style. However, even for the interspecific UI systems, where rejection shows a lower level of specificity, a non-S-RNase could not replace S-RNase.

6. NON-S-RNase FACTORS IN POLLEN REJECTION

S-RNase is the determinant of SI specificity in the pistil and is implicated in interspecific pollen rejection. However, it is only one component of a complex mechanism.

S-RNase-based pollen rejection occurs normally only in the complex extracellular matrix of the pistil. Identifying and characterizing the factors in this matrix that are required for pollen rejection is an area of current research. Much effort has also been devoted to identifying factors that function on the pollen side (Dowd *et al.* 2000). Although candidate pollen genes have been suggested (Lai *et al.* 2002), as yet, none have been definitely implicated.

As more factors (i.e. non-S-RNase factors) are identified in the pollen and pistil, it may be possible to construct an *in vitro* pollen rejection system or devise another approach to investigate the physiology of RNase-based pollen rejection. At this point, there is inadequate knowledge to predict whether soluble components will suffice or whether it will be necessary to construct an artificial matrix analogous to the adhesion system developed to support lily pollen tube growth (Jauh *et al.* 1997). However, efforts to reconstruct S-RNase-based pollen rejection with soluble components have had limited success (Jahnen *et al.* 1989). This suggests that the factors required for pollen rejection are either not soluble, function only at high concentration or function only when assembled into a matrix.

There is considerable genetic evidence that non-S-RNase factors are required for pollen rejection in *Nicotiana*, *Lycopersicon* and *Petunia* (Anderson & de Winton 1931; Mather 1943; Martin 1968; Bernatzky *et al.* 1995). Although most studies address factors required for SI rather than interspecific incompatibility, they do provide useful insights into the nature of the factors involved. It is possible that some of the same factors will function in both SI and interspecific pollen rejection. Some non-S-RNase factors will be simply required for the proper expression of S-RNase. We refer to these as group 1 factors (i.e. factors required for expression of specificity determinants; McClure *et al.* 2000). Tsukamoto *et al.* (1999) described a Uruguayan population of *P. axillaris* that segregates for SI and SC. The defect is attributable to a group 1 factor that affects the expression of S_{13}-RNase. The gene is clearly a non-S-RNase factor because it is not closely linked to the *S*-locus. Bernatzky *et al.* (1995) showed that S-RNase from SI *L. hirsutum* was expressed in backcrossed progeny with SC *L. esculentum*. Despite this, the progeny were SC, demonstrating a requirement for a different kind of non-S-RNase factor. Such factors that are required for pollen rejection but not for S-RNase expression *per se* are referred to as group 2 factors.

The transgenic plant studies discussed here provide evidence that group 2 factors are required for SI and UI in *Nicotiana* (Murfett *et al.* 1996). *N. plumbaginifolia* plants expressing S-RNase from *N. alata* do not display *S*-allele-specific pollen rejection and fail to reject *N. plumbaginifolia* pollen. However, both types of pollen rejection function normally when the transgene is expressed in conjunction with factors from SC *N. alata*.

Loss of a group 1 or a group 2 factor leads to failure of pollen rejection and results in a SC phenotype. Such factors are, therefore, not required for pollen tube growth *per se* and are readily amenable to genetic analysis. Group 3 factors are required for pollen rejection, but also have other roles such as forming a structural component of the extracellular matrix or pollen tube nutrition. Thus, they

are less amenable to genetic analysis because their effects are pleiotropic.

Mutations in any critical factor required for SI will lead to SC (Stone 2002). As SI is linked to interspecific pollen rejection, such mutations could easily affect gene flow between species as well. Moreover, RNase-based SI is widespread but sporadic in the angiosperms, studying the causes of loss of SI may be a useful approach to identifying required factors (Igic & Kohn 2001; Stone 2002).

7. HT PROTEINS

Kondo *et al.* (2002*a*) investigated the mechanisms causing loss of SI in *Lycopersicon*. SI is regarded as the ancestral condition in the genus, and most of the SC taxa form a distinct clade. The SC taxa showed little or no S-RNase expression (Kondo *et al.* 2002*a*). However, different taxa had distinct S-RNase genes and different underlying reasons for low S-RNase expression. The absence of a single type of defect suggests that changing S-RNase expression was not the cause of the shift from SI to SC in the ancestor of the SC taxa. However, all the SC *Lycopersicon* taxa failed to express *HT-B*, a non-S-RNase group 2 factor first identified in *Nicotiana* (Kondo *et al.* 2002*a*; McClure *et al.* 1999). SC *Lycopersicon* taxa possess mutated *HT-B* genes and it is possible that these mutations represent the principal route to SC in *Lycopersicon* (Kondo *et al.* 2002*a*).

HT was originally identified in a differential screen to identify sequences expressed in *N. alata* but not in SC *N. plumbaginifolia* (McClure *et al.* 1999). The sequence cloned from *N. alata* encodes a 101 residue polypeptide with an unusual stretch of asparagine and aspartate residues (i.e. the ND domain) near the C-terminus. A CXXCXC motif is present upstream of the ND domain, and the sequence CXXXCC forms the extreme C-terminus. The functions of these cysteine motifs are unknown. Anti-HT antibodies detect several small polypeptides in style extracts. N-terminal sequencing shows that the major band corresponds to the predicted signal sequence cleavage site. Smaller bands correspond to internal sequences, suggesting the protein is either processed or degraded in style extracts.

HT expression was found to coincide very closely with the developmental onset of SI (McClure *et al.* 1999). In SI *N. alata* $S_{C10}S_{C10}$ flowers can be bud-selfed when they are 2.5 cm long. At this stage S_{C10}-RNase has already accumulated to *ca.* 60% of its maximal level but HT expression is only *ca.* 5% of maximum. A day later, when HT expression is about eightfold higher, bud-selfing is never successful. Antisense suppression of HT prevents S-allele-specific pollen rejection. Antisense plants with undetectable levels of HT showed S_{C10}-RNase expression within normal levels, but failed to reject S_{C10} pollen.

HT-like genes have now been identified in many *Lycopersicon* and *Solanum* species (Kondo *et al.* 2002*b*; O'Brien *et al.* 2002). These plants possess two very tightly linked HT-like genes, *HT-A* and *HT-B*. The *Nicotiana* sequence is most similar to the *HT-B* type. There is no evidence for an *HT-A*-like gene in *N. alata* (B. A. McClure, unpublished data). *HT-A* and *HT-B* in *Lycopersicon* and *Solanum* are more similar to each other than they are to the *N. alata* sequence. This suggests a gene dupli-

cation event in the lineage leading to *Lycopersicon* and *Solanum* or loss of the *HT-A* gene in *Nicotiana*.

O'Brien *et al.* (2002) used antisense and RNAi to test whether *HT-A* and *HT-B* are required for pollen rejection in *S. chacoense*. An antisense *HT-A* construct was introduced into SI *S. chacoense* $S_{12}S_{14}$ plants. Several transformants showed reduced or undetectable levels of *HT-A* transcript; all plants showed normal S-allele-specific pollen rejection. An *HT-B* RNAi construct was also introduced into SI *S. chacoense* $S_{12}S_{14}$. Two plants with low or undetectable levels of *HT-B* transcript set seed when tested with pollen from $S_{12}S_{14}$ plants. The results were similar to those obtained in *Nicotiana* where a change in pollination phenotype was only observed when HT expression was reduced to very low or undetectable levels (McClure *et al.* 1999). The conclusion from the *S. chacoense* study was that *HT-B* is required for S-allele-specific pollen rejection but *HT-A* is not.

O'Brien *et al.* (2002) noted a negative correlation between *HT-B* expression and floral longevity. They suggest that HT-B expression may hasten floral senescence after pollination. Suppression of HT-B could then allow pollen tubes longer to reach the ovary. However, in the *HT*-antisense experiment in *Nicotiana*, compatibility was assessed by using style squashes at a fixed time after pollination (McClure *et al.* 1999). S_{C10} and S_{105} pollen tubes reached the base of the style at about the same time in the antisense plants. Thus, though the HT protein may affect senescence, this is not required to explain the change in pollination behaviour.

As more HT-like gene sequences are determined, sequence analysis may provide clues about function. All the HT-like sequences identified so far possess an ND domain near the C-terminus. However, apart from the preponderance of asparagine and aspartate residues, the ND sequences themselves are not conserved. This suggests a structural role. The ND domains of all the HT-like genes are flanked by the same pattern of cysteine residues (i.e. CXXCXC and CXXXCC) found in the original *N. alata* HT-B sequence. Interestingly, the signal peptide shows the greatest sequence conservation when HT-A and HT-B proteins are compared across *Lycopersicon*, *Solanum* and *Nicotiana* (Cruz-Garcia *et al.* 2003). This may suggest that the processing of HT proteins is important. Finally, as *HT-B* genes function in S-allele-specific pollen rejection but *HT-A* genes apparently do not, the sequence differences between them may be informative. The most obvious difference is that HT-B proteins possess a sequence similar to PSISLL near the N-terminus of the mature protein that is missing from the HT-A class.

8. STYLAR GLYCOPROTEINS AND POLLEN REJECTION

Although S-RNase and HT-B proteins are the only factors directly implicated in RNase-based pollen rejection, other factors are likely to be involved. One hypothesis is that such factors may interact with S-RNase in the stylar matrix. To test this hypothesis S_{C10}-RNase was immobilized on Affigel and used as an affinity resin. When extracts from SI *N. alata* $S_{C10}S_{C10}$ were analysed, transmitting-specific glycoprotein (first characterized in *N. tabacum*)

was identified as a major S-RNase binding protein (McClure *et al.* 2000).

TTS is an AGP that accumulates in the stylar extracellular matrix of *N. tabacum* and *N. alata* (Cheung *et al.* 1993, 1995; Wu *et al.* 1995, 2000). The protein is thought to support compatible pollen tube growth because it enhances pollen tube growth *in vitro*, and antisense inhibition of TTS expression in *N. tabacum* interferes with pollen tube growth *in vivo*. Immunolocalization studies show the polypeptide associated with the pollen tube cell membrane. Antisense TTS plants also show altered style morphology, suggesting that TTS also plays a structural role. The fact that TTS binds to S-RNase indicates that it may have a third role in SI *N. alata* and act as a group 3 SI factor.

TTS is a member of a family of AGPs that interact with pollen tubes. The mature TTS polypeptide consists of two domains. The N-terminal domain is proline rich and is decorated with arabinogalactan. The C-terminal 137 amino acids contain six cysteine residues and may form a distinct globular domain. Similar C-terminal domains occur in the 120 kDa glycoprotein from *N. alata* (Lind *et al.* 1994; Schultz *et al.* 1997) and in the PELPIII from *N. tabacum* (de Graaf 1999). The last two proteins are also abundant stylar AGPs that interact with pollen tubes. Immunolocalization studies show that the 120 kDa glycoprotein is taken up into the pollen tube cytoplasm in *N. alata* (Lind *et al.* 1996). Similar immunolocalization studies in *N. tabacum* show that PELPIII polypeptide associates with the pollen tube callose wall and callose plugs (de Graaf 1999).

It has long been thought that AGPs may serve as recognition molecules in the style (Harrison *et al.* 1984). TTS, the 120 kDa glycoprotein, and PELPIII are good candidates for such a function because they form a major fraction of the stylar matrix and they are known to interact with pollen tubes. If these proteins are involved in pollen recognition at species level they would be expected to show sequence polymorphism. This hypothesis could be tested by examining TTS (120 kDa) and PELPIII homologues from different species. Polymorphisms that correlate with inter- or intraspecific pollination behaviour would support the idea that these proteins play a part in controlling pollination.

9. PROSPECTS

There is now a clear link between SI and UI. However, it is also clear that UI is complex. Not only is the *S*-locus implicated in different UI systems in different ways, it is also clear that *S*-locus-independent mechanisms also contribute to UI. This complexity explains, at least in part, the controversy over the role of the *S*-locus in UI. The linkage between SI and UI offers opportunities to unravel the molecular mechanisms of pollen rejection. Each system offers different experimental approaches to identify the functional components. Studies in one system will help other studies, thus improving the prospects for a detailed understanding.

REFERENCES

Anderson, E. & de Winton, D. 1931 The genetic analysis of an unusual relationship between self-sterility and self-fertility in *Nicotiana*. *A. Missouri Bot. Garden* **18**, 97–116.

Anderson, M. A. (and 17 others) 1986 Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. *Nature* **321**, 38–44.

Beecher, B. 2001 RNases and interspecific pollen rejection. PhD thesis, University of Missouri-Columbia, MO, USA.

Beecher, B. & McClure, B. A. 2001 Effects of RNases on rejection of pollen from *Nicotiana tabacum* and *N. plumbaginifolia*. *Sex. Pl. Reprod.* **14**, 69–76.

Beecher, B., Murfett, J. & McClure, B. A. 1998 RNaseI from *E. coli* cannot substitute for S-RNase in rejection *Nicotiana plumbaginifolia* pollen. *Pl. Mol. Biol.* **36**, 553–563.

Bernacchi, D. & Tanksley, S. D. 1997 An interspecific cross of *Lycopersicon esculentum* × *L. hirsutum*: linkage analysis and a QTL analysis of sexual compatibility factors and floral traits. *Genetics* **147**, 861–877.

Bernatzky, R., Glaven, R. H. & Rivers, B. A. 1995 S-related protein can be recombined with self-compatibility in interspecific derivatives of *Lycopersicon*. *Biochem. Genet.* **33**, 215–225.

Bredemeijer, G. M. M. & Blaas, J. 1981 S-Specific proteins in styles of self-incompatible *Nicotiana alata*. *Theor. Appl. Genet.* **59**, 185–190.

Chetelat, R. T. & DeVerna, J. W. 1991 Expression of unilateral incompatibility in pollen of *Lycopersicon pennellii* is determined by major loci on chromosomes 1, 6 and 10. *Theor. Appl. Genet.* **82**, 704–712.

Cheung, A. Y. 1996 The pollen tube growth pathway: its molecular and biochemical contributions and responses to pollination. *Sex. Pl. Reprod.* **9**, 330–336.

Cheung, A. Y., May, B., Kawata, E., Gu, Q. & Wu, H.-m. 1993 Characterization of cDNAs for stylar transmitting tissue-specific proline-rich proteins in tobacco. *Pl. J.* **3**, 151–160.

Cheung, A. Y., Wang, H. & Wu, H.-m. 1995 A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. *Cell* **82**, 383–393.

Cruz-Garcia, F., Hancock, C. N. & McClure, B. 2003 S-RNase complexes and pollen rejection. *J. Exp. Bot.* **59**, 123–130.

de Graaf, B. H. J. 1999 Pistil proline-rich proteins in *Nicotiana tabacum*. PhD thesis, Catholic University, Nijmegen, The Netherlands.

de Nettancourt, D. 1997 Incompatibility in angiosperms. *Sex. Pl. Reprod.* **10**, 185–199.

de Nettancourt, D. 2001 *Incompatibility and incongruity in wild and cultivated plants*, 2nd edn. Berlin: Springer.

Dowd, P. E., McCubbin, A. G., Wang, X., Verica, J. A., Tsukamoto, T., Ando, T. & Kao, T.-H. 2000 Use of *Petunia inflata* as a model for the study of solanceous type self-incompatibility. *Ann. Bot.* **85**, 87–93.

Gray, J. E., McClure, B. A., Bönig, I., Anderson, M. A. & Clarke, A. E. 1991 Action of the style product of the self-incompatibility gene of *Nicotiana alata* (S-RNase) on *in vitro*-grown pollen tubes. *Pl. Cell* **3**, 271–283.

Harrison, P. J., Anderson, M. A., Bacic, A. & Clarke, A. E. 1984 *Cell–cell recognition in plants with special reference to the pollen–stigma interaction*, pp. 161–203. Oxford University Press.

Hiscock, S. J. & Dickinson, H. G. 1993 Unilateral incompatibiltiy within the Brassicaceae: further evidence for the involvement of the self-incompatibility (S)-locus. *Theor. Appl. Genet.* **86**, 744–753.

Hogenboom, N. G. 1984 Incongruity: non-functioning of intercellular and intracellular partner relationships through

non-matching information. In *Encyclopedia of plant physiology* (ed. H. F. Linskens & J. Heslop-Harrison), pp. 640–654. Berlin: Springer.

Huang, S., Lee, H.-S., Karunanandaa, B. & Kao, T.-H. 1994 Ribonuclease activity of *Petunia inflata* S proteins is essential for rejection of self-pollen. *Pl. Cell* **6**, 1021–1028.

Igic, B. & Kohn, J. R. 2001 Evolutionary relationships among self-incompatibility RNases. *Proc. Natl Acad. Sci. USA* **98**, 13 167–13 171.

Ioerger, T. R., Gohlke, J. R., Xu, B. & Kao, T.-H. 1991 Primary structural features of the self-incompatibility protein in Solanaceae. *Sex. Pl. Reprod.* **4**, 81–87.

Jahnen, W., Lush, W. M. & Clarke, A. E. 1989 Inhibition of *in vitro* pollen tube growth by isolated S-glycoproteins of *Nicotiana alata*. *Pl. Cell* **1**, 501–510.

Jauh, G. Y., Eckard, K. J., Nothnagel, E. A. & Lord, E. M. 1997 Adhesion of lily pollen tubes on an artificial matrix. *Sex. Pl. Reprod.* **10**, 173–180.

Kawata, Y., Sakiyama, F. & Tamaoki, H. 1988 Amino acid sequence of ribonuclease T2 from *Aspergillus oryzae*. *Eur. J. Biochem.* **176**, 683–697.

Kondo, K., Yamamoto, M., Itahashi, R., Sato, T., Egashira, H., Hattori, T. & Kowyama, Y. 2002*a* Insights into the evolution of self-compatibility in *Lycopersicon* from a study of stylar factors. *Pl. J.* **30**, 143–153.

Kondo, K., Yamamoto, M., Matton, D. P., Sato, T., Masashi, H., Norioka, S., Hattori, T. & Kowyama, Y. 2002*b* Cultivated tomato has defects in both S-RNase and HT genes required for stylar function of self-incompatibility. *Pl. J.* **29**, 627–636.

Lai, Z., Ma, W., Han, B., Liang, L., Zhang, Y., Hong, G. & Xue, Y. 2002 An F-box gene linked to the self-incompatibility (S) locus of *Antirrhinum* is expressed specifically in pollen and tapetum. *Pl. Mol. Biol.* **50**, 29–42.

Lewis, D. & Crowe, L. K. 1958 Unilateral interspecific incompatibility in flowering plants. *Heredity* **12**, 233–256.

Lind, J. L., Bacic, A., Clarke, A. E. & Anderson, M. A. 1994 A style-specific hydroxyproline-rich glycoprotein with properties of both extensins and arabinogalactan proteins. *Pl. J.* **6**, 491–502.

Lind, J. L., Bönig, I., Clarke, A. E. & Anderson, M. A. 1996 A style-specific 120 kDa glycoprotein enters pollen tubes of *Nicotiana alata in vivo*. *Sex. Pl. Reprod.* **9**, 75–86.

Lush, W. M. & Clarke, A. E. 1997 Observations of pollen tube growth in *Nicotiana alata* and their implications for the mechanism of self-incompatibility. *Sex. Pl. Reprod.* **10**, 27–35.

Lush, W. M., Grieser, F. & Wolters-Arts, M. 1998 Directional guidance of *Nicotiana alata* pollen tubes *in vitro* and on the stigma. *Pl. Physiol.* **118**, 733–741.

McClure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Simpson, R. J., Sakiyama, F. & Clarke, A. E. 1989 Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* **342**, 955–957.

McClure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Bacic, A. & Clarke, A. E. 1990 Molecular genetics and biology of self-incompatibility in *Nicotiana alata*, an ornamental tobacco. *Aust. J. Pl. Physiol.* **17**, 345–353.

McClure, B. A., Mou, B., Canevascini, S. & Bernatzky, R. 1999 A small asparagine-rich protein required for S-allele-specific pollen rejection in *Nicotiana*. *Proc. Natl Acad. Sci. USA* **96**, 13 548–13 553.

McClure, B. A., Cruz-Garcia, F., Beecher, B. S. & Sulaman, W. 2000 Factors affecting inter- and intra-specific pollen rejection in *Nicotiana*. *Ann. Bot.* **85**, 113–123.

Martin, F. W. 1967 The genetic control of unilateral incompatibility between two tomato species. *Genetics* **56**, 391–398.

Martin, F. W. 1968 The behaviour of *Lycopersicon* incompatibility alleles in an alien genetic milieu. *Genetics* **60**, 101–109.

Mather, K. 1943 Specific differences in *Petunia* I. Incompatibility. *J. Genet.* **45**, 215–235.

Murfett, J., Atherton, T. L., Mou, B., Gasser, C. S. & McClure, B. A. 1994 S-RNase expressed in transgenic *Nicotiana* causes S-allele-specific pollen rejection. *Nature* **367**, 563–566.

Murfett, J. M., Strabala, T. J., Zurek, D. M., Mou, B., Beecher, B. & McClure, B. A. 1996 *S* RNase and interspecific pollen rejection in the genus *Nicotiana*: multiple pollen rejection pathways contribute to unilateral incompatibility between self-incompatible and self-compatible species. *Pl. Cell* **8**, 943–958.

O'Brien, M., Kapfer, C., Major, G., Laurin, M., Bertrand, C., Kondo, K., Kowyama, Y. & Matton, D. P. 2002 Molecular analysis of the stylar-expressed *Solanum chacoense* asparagine-rich protein family related to the HT modifier of gametophytic self-incompatibility in *Nicotiana*. *Pl. J.* **32**, 985–996.

Pandey, K. K. 1973 Phases in the *S*-gene expression, and *S*-allele interaction in the control of interspecific incompatibility. *Heredity* **31**, 381–400.

Schultz, C. J., Hauser, K., Lind, J. L., Atkinson, A. H., Pu, Z. Y., Anderson, M. A. & Clarke, A. E. 1997 Molecular characterization of a cDNA sequence encoding the backbone of a style-specific 120 kDa glycoprotein which has features of both extensins and arabinogalactan proteins. *Pl. Mol. Biol.* **35**, 833–845.

Stone, J. L. 2002 Molecular mechanisms underlying the breakdown of gametophytic self-incompatibility. *Q. Rev. Biol.* **77**, 17–32.

Tsukamoto, T., Ando, T., Kokubun, H., Watanabe, H., Masada, M., Zhu, X., Marchesi, E. & Kao, T.-H. 1999 Breakdown of self-incompatibility in a natural population of *Petunia axillaris* (Solanaceae) in Uruguay containing both self-incompatible and self-compatible plants. *Sex. Pl. Reprod.* **12**, 6–13.

Walles, B. & Han, S. P. 1998 Ribosomes in incompatible pollen tubes in the Solanaceae. *Physiol. Pl.* **103**, 461–465.

Wu, H.-m., Wang, H. & Cheung, A. Y. 1995 A pollen tube growth stimulatory glycoprotein is deglycosylated by pollen tubes and displays a glycosylation gradient in the flower. *Cell* **83**, 395–403.

Wu, H.-m., Wong, E., Ogdahl, J. & Cheung, A. Y. 2000 A pollen tube growth promoting arabinogalactan protein from *Nicotiana alata* is similar to the tobacco TTS protein. *Pl. J.* **22**, 165–176.

Zurek, D. M., Mou, B., Beecher, B. & McClure, B. 1997 Exchanging sequence domains between S-RNases from *Nicotiana alata* disrupts pollen recognition. *Pl. J.* **11**, 797–808.

GLOSSARY

AGP: arabinogalactan protein
ND: asparagine/aspartate
PELPIII: class III pistil-specific extension-like protein
SC: self-compatibility
SI: self-incompatibility
TTS: transmitting tract specific
UI: unilateral incompatibility