RESEARCH ARTICLE

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

Jane Murfett, Timothy J. Strabala, Daniel M. Zurek, Beiquan Mou, Brian Beecher, and Bruce A. McClure

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

In self-incompatible (SI) plants, the S locus acts to prevent growth of self-pollen and thus promotes outcrossing within the species. Interspecific crosses between SI and self-compatible (SC) species often show unilateral incompatibility that follows the SI × SC rule: SI species reject pollen from SC species, but the reciprocal crosses are usually compatible. The general validity of the SI × SC rule suggests a link between SI and interspecific pollen rejection; however, this link has been questioned because of a number of exceptions to the rule. To clarify the role of the S locus in interspecific pollen rejection, we transformed several *Nicotiana* species and hybrids with genes encoding S<sub>A</sub> or S<sub>C</sub> RNase from SI *N. alata*. Compatibility phenotypes in the transgenic plants were tested using pollen from three SC species showing unilateral incompatibility with *N. alata*. S RNase was implicated in rejecting pollen from all three species. Rejection of *N. plumbaginifolia* pollen was similar to S allele-specific pollen rejection, showing a requirement for both S RNase and other genetic factors from *N. alata*. In contrast, S RNase-dependent rejection of *N. glutinosa* and *N. tabacum* pollen proceeded without these additional factors. *N. alata* also rejects pollen from the latter two species through an S RNase-independent mechanism. Our results implicate the S locus in all three systems, but it is clear that multiple mechanisms contribute to interspecific pollen rejection.

INTRODUCTION

Many plants have evolved genetically controlled self-incompatibility (SI) systems that promote outcrossing by restricting pollination between closely related individuals of the same species (de Nettancourt, 1977). Mechanisms also exist to restrict pollination between different species, but comparatively little is known about the control of interspecific pollination.

As in other solanaceous plants, SI in the genus *Nicotiana* is controlled by a single multiallelic locus, the S locus (Newbigin et al., 1993). These plants employ a gametophytic SI system in which pollen is rejected if the S allele in the haploid pollen is the same as either S allele in the diploid pistil. S allele-specific pollen rejection occurs as pollen tubes grow through the extracellular matrix of the stylar transmitting tract (Newbigin et al., 1993). The products of the S locus in the style are the S RNases (McClure et al., 1989). These glycoproteins are very abundant in the extracellular matrix of the transmitting tract and are also expressed in the stigma and in the epidermis of the placenta (Cornish et al., 1987; Anderson et al., 1989; McClure et al., 1993). S RNases are essential for S allele-specific pollen rejection (Lee et al., 1994; Murfett et al., 1994), and their ribonuclease activity is required for this function (Huang et al., 1994). Following incompatible pollinations, RNA in self-pollen tubes is degraded, and this degradation is consistent with a cytotoxic model for pollen rejection (McClure et al., 1990; Gray et al., 1991; Dickinson, 1994). SI is therefore an active process in which S RNases determine allelic specificity and probably act directly to inhibit the growth of incompatible pollen tubes.

In contrast, little is known about the biochemical mechanisms controlling interspecific incompatibility, which is defined as a postpollination process preventing the formation of hybrid zygotes "through an absence of pollen germination or an abnormal behavior of pollen tubes" (de Nettancourt, 1977). A common type of interspecific crossing relationship occurs in
families in which both SI and self-compatible (SC) species exist. In interspecific crosses, SI plants usually show unilateral incompatibility (UI) with related SC species. Such crosses often follow the SI × SC rule: the SI pistil rejects pollen from the SC species, but the reciprocal cross is compatible (Lewis and Crowe, 1958; de Nettancourt, 1977). Reciprocal crosses between two related SI species or two related SC species are usually compatible (Lewis and Crowe, 1958). Genetic studies with the Solanaceae (Chetelat and Deverna, 1991) and the Brassicaceae (Hiscock and Dickinson, 1993) have implicated the S locus in this type of interspecific pollen rejection. By using genetic methods, three separate loci, one of which is at or near the S locus, were shown to be involved in interspecific pollen rejection in *Lycopersicon* (Chetelat and Deverna, 1991).

These genetic studies support the hypothesis that the S locus acts to restrict outcrossing at the interspecific level while also preventing self-pollination within species. However, involvement of the S locus in interspecific pollen rejection is controversial because there are exceptions to the SI × SC rule (Hogenboom, 1975, 1984; Mutschler and Leidl, 1994). Moreover, it has been argued that interspecific and intraspecific pollen rejection may occur through separate mechanisms. The rejection reactions typically occur at different positions in the pistil, and inhibited pollen tubes show characteristic differences in morphology (Ascher and Peloquin, 1968; Hogenboom, 1975). Because both SI and unilateral interspecific incompatibility are likely to involve several loci (Martin, 1967, 1968; Ai et al., 1991; Bernatzky et al., 1995), it has been difficult to design purely genetic strategies to test definitively for the sufficiency of the S locus in interspecific pollen rejection. However, by manipulating the expression of cloned S RNase genes in transgenic plants, it is possible to test directly for the involvement of the S locus in interspecific pollen rejection. This approach avoids many of the complications inherent in the earlier genetic studies.

We have used sense and antisense constructs to manipulate expression of *S*$_{A2}$ and *S*$_{D10}$ RNase from *N. alata* in four different genetic backgrounds. Transformed plants were tested for compatibility with pollen from species showing UI with *N. alata*. By determining the effects of the transgenes on pollination behavior in several different backgrounds, it is possible to examine interactions between S RNase and other genetic factors. *N. plumaginifolia* pollen was used to test for the effect of S RNase on a system that follows the SI × SC rule. *N. glutinosa* and *N. tabacum* were used as examples of species that show UI with *N. alata* but do not follow the SI × SC rule. S RNase was implicated in all three UI systems. Thus, in crosses with *N. alata*, the F$_1$ progeny (i.e., *N. alata* cv Breakthrough × *N. alata* S$_5$S$_5$) are self-fertile and reject pollen only from the SI parent (data not shown). Taken together, the biochemical and genetic data suggest that cultivar Breakthrough is SC because it does not express an S RNase. Significantly, this SC accession of *N. alata* behaves differently from SI *N. alata* in interspecific crosses. This accession accepts pollen from *N. plumaginifolia* but rejects pollen from *N. tabacum* and *N. glutinosa*. Thus, in crosses with *N. alata*, *N. plumaginifolia* follows the SI × SC rule, but *N. tabacum* and *N. glutinosa* do not. Clearly, the genetic mechanism for rejecting pollen from the latter species differs from the mechanism for rejecting *N. plumaginifolia* pollen.

Each of the three SC species has a unique protein profile and did not show prominent S RNase–like bands. The pollination results showed that *N. tabacum*, used as transgene recipient in the experiments described below, sets seed after pollination with any of the plants tested here. *N. glutinosa* and *N. plumaginifolia* pistils also support the growth of all types of pollen used in this study. However, in several instances, seed set does not occur (Pandey, 1981). This may be related to differences in chromosome number. With the exception of the *N. plumaginifolia* × *N. glutinosa* cross, pollen tube morphologies appeared normal when examined in aniline blue–stained style squashes. In this cross, pollen tubes reached the base of the style within 48 hr, but the morphology was somewhat abnormal (data not shown).

**S RNase Gene Constructs and Plant Transformation**

The gene constructs used in this study are shown in Figure 2A. The expression constructs pSA29617 and pSA2S13 (Figure 2A) include the entire *S*$_{A2}$ RNase coding region.
interrupted by a 114-bp intron and 2.5 kb of 3' sequence. The tomato chitinase ChiP and S locus glycoprotein SLG gene promoters have been shown to provide high-level expression in mature pistils (Dzelzkalns et al., 1993; Gasser et al., 1993; Murfett et al., 1994). The pSC109617 construct is similar to pSA29617, except that the region encoding the S2 RNase mature protein and the intron is replaced with the corresponding regions from the SC10 RNase gene. The antisense constructs pHAKA and pBAGA were designed to suppress S2 RNase expression (Figure 2A). A comparison was made between the efficiency of a tissue-specific promoter (i.e., the ChiP promoter in pHAKA) and a nonspecific promoter (i.e., the cauliflower mosaic virus 3S promoter in pBAGA) for antisense suppression of S2 RNase expression. Plants carrying the pBAGA construct showed suppression of S2 RNase expression most frequently, although plants containing pHAKA also showed suppression (Murfett et al., 1995).

The most straightforward test of the hypothesis that S RNase causes rejection of pollen from SC species is to introduce a cloned gene directly into an SC genetic background and test for a new pollination phenotype. We employed two such SC genetic backgrounds. As diagrammed in Figure 2B, the S2 RNase expression construct pSA29617 was introduced into N. tabacum, and the pollination phenotype was tested in second generation–transformed plants. N. tabacum was also transformed with pSC109617 to determine whether altered pollination phenotypes could be obtained with a second S RNase (see below). However, in this case, the pollination phenotypes were determined with primary transformants.

Because genetic studies suggest a requirement for multiple loci in both interspecific pollen rejection and SI (Martin, 1967, 1968; Ai et al., 1991; Chetelat and DeVerna, 1991), experiments also were designed to examine potential interactions between S RNase and genetic factors that might be absent from an SC background (Figure 2C). N. alata itself is difficult to transform because of its poor regeneration efficiency (Ebert and Clarke, 1990). However, SC N. plumbaginifolia can be transformed easily, and non–S RNase factors from the N. alata background can be supplied in trans by forming (N. plumbaginifolia × N. alata) hybrids. As shown in Figure 2C, N. plumbaginifolia plants were transformed with S2 RNase expression constructs pSA29617 and pSA2S13. Primary transformants were then crossed with untransformed N. plumbaginifolia to observe the pollination behavior of plants expressing S2 RNase in a second SC genetic background (Figure 2C, center). Some of the same primary transformants were also crossed with SC N. alata cv Breakthrough to generate transgenic (N. plumbaginifolia × SC N. alata) hybrids (Figure 2C, center). These experiments allowed us to compare the pollination behavior of plants expressing S2 RNase in purely SC genetic backgrounds (i.e., N. plumbaginifolia and N. tabacum) with the behavior of the same transgene in combination with other genetic factors from the N. alata background (i.e., in [N. plumbaginifolia × SC N. alata] hybrids). In planning these experiments, we assumed that additional genetic factors that might be needed for S RNase function would be present in N. alata cv Breakthrough. This assumption was borne out by later results.

In these genetic backgrounds, the pollination behavior of untransformed controls can be compared with that of transformed plants expressing S RNase. To determine whether changes in pollination behavior have a specific requirement for S RNase, N. plumbaginifolia was also transformed with
A

**Sense-S\textsubscript{A2} constructs**

- pSA29617: Chip promoter \( S\textsubscript{A2} \) coding + intron \( S\textsubscript{A2} 3' \)
- pSA2S13: B19 promoter \( S\textsubscript{A2} \) coding + intron \( S\textsubscript{A2} 3' \)

**Sense-S\textsubscript{C10} construct**

- pSC109617: Chip promoter \( S\textsubscript{C10} \) coding + intron \( S\textsubscript{A2} 3' \)

**Antisense-S\textsubscript{A2 constructs**

- pHAKA: Chip promoter \( S\textsubscript{A2} \) cDNA NOS 3'
- pBAGA: 3SS promoter \( S\textsubscript{A2} \) cDNA NOS 3'

B

**Construct**

- N. tabacum
  - Sense-S\textsubscript{A2} RNase in N. tabacum Background
  - Sense-S\textsubscript{C10} RNase in N. tabacum Background

C

**Construct**

- N. plumbaginifolia
  - Sense-S\textsubscript{A2} RNase in N. plumbaginifolia Background
  - Antisense-S\textsubscript{A2} RNase in Hybrid Background

**Outcross Parent**

- N. plumbaginifolia
- SC N. alata
- N. alata

Figure 2. Experimental Strategy.

(A) \( S\textsubscript{A2} \) RNase gene constructs. Expression constructs include the *N. alata* S RNase gene coding region, interrupted by a small intron, and 2.5 kb of 3' sequence from the *N. alata* \( S\textsubscript{A2} \) RNase gene (SA2 3'). pSA29617 includes the 1.2-kb tomato ChiP promoter described previously (Murfett et al., 1994). pSA2S13 includes the 3.65-kb Brassica SLG13 promoter (Dzelzkalns et al., 1993). pSC109617 is similar to pSA29617, except it contains the coding region and intron from the *N. alata* \( S\textsubscript{C10} \) RNase gene. The antisense constructs contain 550 bp from the S' end of the \( S\textsubscript{A2} \) cDNA, followed by the nopaline synthase polyadenylation sequence (NOS 3'; Bevan et al., 1983). Antisense transcription was driven by either the tomato ChiP promoter in pHAKA or the cauliflower mosaic virus 3SS promoter with duplicated enhancer (Kay et al., 1987) in pBAGA.

(B) Expression of S RNase in *N. tabacum*. Plants were transformed with either \( S\textsubscript{A2} \) or \( S\textsubscript{C10} \) RNase expression constructs. Primary transformants expressing \( S\textsubscript{A2} \) RNase were outcrossed, and pollenation phenotypes were determined in the progeny (shown in Figure 3A). pSC109617 transformants were analyzed directly (shown in Figure 3B).

(C) Crossing scheme for N. plumbaginifolia transformants. *N. plumbaginifolia* primary transformants were outcrossed, and the effects of \( S\textsubscript{A2} \) RNase gene expression on pollen-rejection phenotypes were determined in three different genetic backgrounds. Thus, interaction between \( S\textsubscript{A2} \) RNase and the purely SC \( S\textsubscript{A2} \) RNase was observed when the antisense transgene was crossed into the *N. alata* \( S\textsubscript{A2} \) RNase genetic background. Antisense transgene expression on pollen-rejection phenotypes were determined in three different genetic backgrounds. Thus, interaction between \( S\textsubscript{A2} \) RNase and the purely SC \( S\textsubscript{A2} \) RNase was observed when the antisense transgene was crossed into the *N. alata* \( S\textsubscript{A2} \) RNase genetic background. Both (*N. plumbaginifolia* × SC *N. alata cv Breakthrough*) and (*N. plumbaginifolia* × SI *N. alata* \( S\textsubscript{A2} \) RNase) hybrids are extremely vigorous. However, perhaps due to differences in chromosome numbers between *N. plumbaginifolia* and *N. alata*, the hybrids are sterile. Nonetheless, prezygotic fertilization barriers operate as usual in these plants. For example, (*N. plumbaginifolia* × SI *N. alata* \( S\textsubscript{A2} \) RNase) hybrids reject pollen from *N. alata* \( S\textsubscript{A2} \) RNase but not from *N. alata* \( S\textsubscript{C10} \) RNase (Murfett et al., 1995). Furthermore, as described below, untransformed (*N. plumbaginifolia* × SI *N. alata* \( S\textsubscript{A2} \) RNase) hybrids reject pollen from *N. plumbaginifolia*, whereas (*N. plumbaginifolia* × SC *N. alata*) do not. The ability of SC *N. alata* cv Breakthrough to reject pollen from *N. glutinosa* and *N. tabacum* acts as a dominant trait, and untransformed (*N. plumbaginifolia* × SC *N. alata*) hybrids reject pollen from these SC species. The pollination behavior of the untransformed hybrids is therefore a straightforward extrapolation of the behavior of the parental species. These hybrids, along with the three SC species, provide a convenient system to determine the effects of S RNase on pollination behavior.

S RNase Expression in Transgenic Plants

Stylar RNase specific activity and immunoblotting were used to monitor S RNase expression levels in the transgenic plants. With the exception of the (*N. plumbaginifolia* × SI *N. alata* × *N. glutinosa*) hybrid, expression of S RNase was observed even in the *N. plumbaginifolia* primary transformants. Antisense constructs were directed by either the tomato ChiP promoter in pHAKA or the cauliflower mosaic virus 35S promoter with duplicated enhancer (Kay et al., 1987) in pBAGA. Antisense transcription was driven by either the tomato ChiP promoter in pHAKA or the cauliflower mosaic virus 35S promoter with duplicated enhancer (Kay et al., 1987) in pBAGA. Antisense transcription was driven by either the tomato ChiP promoter in pHAKA or the cauliflower mosaic virus 35S promoter with duplicated enhancer (Kay et al., 1987) in pBAGA.
Figure 3. S RNase Expression in SC Genetic Backgrounds.

Shown are stylar RNase specific activities of transformed and control plants. Cross-hatched bars show the RNase specific activity of extracts from N. alata S₂A₂S₂ (A) and N. glutinosa (B) homozygotes. The white bars show the RNase specific activity of untransformed controls (N. tabacum in [A] and [B] or N. plumbaginifolia in [C]). Hatched bars show RNase specific activities of style extracts from individual transgenic plants. Style proteins were also separated by SDS-PAGE, and immunoblot analysis was performed using antibodies specific for S₂.

Interspecific Pollen Rejection 947

Although non-S RNases are also expressed in the style (McCulure et al., 1989; Lee and Kao, 1992), the contribution of these enzymes to total stylar RNase specific activity would be expected to be relatively constant in control and transformed plants. The activity of the transgenes is therefore reflected in a change in stylar RNase specific activity of the transformed plants compared with the controls. Many of the plants transformed with S RNase expression constructs showed stylar RNase specific activities comparable to those of N. alata S homozygotes (Figures 3A to 3C, and 4A). Several plants transformed with antisense S₂A₂ RNase constructs showed stylar RNase specific activities comparable to those of untransformed SC plants (Figures 4A and 4B). Within each group, a range of expression levels was observed. This type of variation in gene expression levels is typical in plant transformation.

S₂A₂S₂A₂ hybrid, the plants used in this study possess relatively low endogenous RNase activity. In Figures 3 and 4, stylar RNase specific activity has therefore been used as a convenient measure of transgene expression level. Plants transformed with S RNase expression constructs showed increased stylar RNase activity when compared with controls (Figure 3A to 3C, and 4A), and antisense suppression of S RNase led to a decrease in stylar RNase activity (Figure 4B).

The allelic S RNases show a remarkable variation in specific activity (McCulure et al., 1989). Purified S₂A₂ RNase has one of the lowest (i.e., 83 A₂₆₀ units min⁻¹ mg⁻¹) and purified S₇C₁₀ RNase has one of the highest (i.e., 4000 A₂₆₀ units min⁻¹ mg⁻¹) specific activities reported. Thus, the stylar RNase specific activities in the transformed plants vary widely, depending on whether the plants express S₂A₂ or S₇C₁₀ RNase (e.g., Figure 3A versus Figure 3B).

Pollinations were performed with untransformed N. tabacum, N. plumbaginifolia (N. plumbag.), N. glutinosa, and SI N. alata S₂A₂S₂A₂ or S₇C₁₀S₇C₁₀. Pollination results are presented as the number of compatible pollinations over the total number of pollinations attempted. All available data are presented. In some cases, only a single pollination was performed; in these cases, the phenotypes are tentative. All compatible pollinations resulted in seed set. Data sets that indicate a change in pollination phenotype due to high-level expression of the transgene are underlined. nd, no data.

(A) Transgenic N. tabacum plants expressing S₂A₂ RNase. Progeny derived from two independent N. tabacum plants transformed with pSA29617 (i.e., plants 4 and 5) are grouped.

(B) Transgenic N. tabacum plants expressing S₇C₁₀ RNase. Data are shown for six independent N. tabacum transformants containing the pSC109617 construct.

(C) Transgenic N. plumbaginifolia plants expressing S₂A₂ RNase. Progeny from two independent pSA29617 transformants and three independent pSA2S13 transformants are grouped.
used to confirm that changes in stilar RNase specific activity are due to changes in S RNase expression level. These antibodies do not cross-react with non-S RNases present in styles of untransformed controls (Figures 3 and 4). S A2 RNase expressed in the transgenic plants was observed to comigrate in SDS–polyacrylamide gels with S A2 RNase expressed in N. alata (Figures 3A, 3B, 4A, and 4B). However, multiple S C10 RNase bands were observed in N. tabacum plants transformed with pSC109617 (Figure 3B). The origin of this heterogeneity is not known but could be related to a post-translational modification such as differential N-glycosylation. Both S A2 and S C10 RNase expressed in N. tabacum are active RNase enzymes, and both are active in rejection of N. glutinosa and N. tabacum pollen (see below).

**S Allele-Specific Pollen Rejection Does Not Occur in the Purely SC Backgrounds** **N. tabacum and N. plumbaginifolia**

The pollination data in Figures 3A to 3C show that S allele-specific pollen rejection does not occur in the N. tabacum and N. plumbaginifolia genetic backgrounds, regardless of S RNase expression level. Figure 3A shows S A2 RNase expression in progeny N. tabacum plants derived from two independent pSA29617 transformants. Figure 3B shows data for six N. tabacum transformants expressing S C10 RNase. Figure 3C shows data for S A2 RNase expression in N. plumbaginifolia, including progeny of two independent pSA29617 transformants and three independent pSA2S13 transformants. Although several plants express S A2 or S C10 RNase at levels comparable to an N. alata S homozygote, they all accept pollen from SI N. alata genotypes S A2S A2 and S C10S C10. Therefore, in these genetic backgrounds, S A2 RNase does not cause rejection of N. alata S A2 pollen, and S C10 RNase does not cause rejection of S C10 pollen. However, Figure 4A shows that in transgenic (N. plumbaginifolia × SC N. alata) progeny, high-level S A2 RNase expression causes rejection of S A2 but not S C10 pollen. Untransformed control (N. plumbaginifolia × SC N. alata) hybrids accept pollen of both genotypes. Thus, S allele-specific pollen rejection occurs when S A2 RNase is expressed in hybrids with SC N. alata cv Breakthrough. Similar results obtained using a transgenic (N. langsdorffii × SC N. alata) hybrid (Murfett et al., 1994) have been described previously.

Studies with transgenic plants (Murfett et al., 1992, 1994) and with untransformed materials (Clark et al., 1999) suggest that a high threshold level of S RNase expression is required for pollen rejection. As shown in Figure 4A, plants that express S A2 RNase at moderate levels reject S A2 pollen in some but not all crosses. The data in Figure 4B confirm that, as expected, antisense inhibition of S A2 RNase expression specifically suppresses the ability to reject S A2 pollen (see also Murfett et al., 1995). Similar results have been reported in Petunia inflata (Lee et al., 1994). Transgenic (N. plumbaginifolia × SI N. alata S A2S A2) hybrids in which S A2 RNase expression has been...
strongly suppressed accept both \( S_A^2 \) and \( S_{C10} \) pollen. Untransformed control hybrids and unsuppressed hybrids specifically reject \( N. \text{alata} \) \( S_A^2 \) pollen (Figure 4B). Thus, the stylar incompatibility phenotypes of the \((N. \text{plumbaginifolia} \times \text{N. alata})\) hybrids are similar to the \( N. \text{alata} \) parent.

S RNase Expression and Rejection of \( N. \text{plumbaginifolia} \) Pollen

As for \( S \) allele–specific pollen rejection, the data in Figures 3A to 3C show that S RNase expression in \( N. \text{tabacum} \) and \( N. \text{plumbaginifolia} \) does not cause rejection of pollen from untransformed \( N. \text{plumbaginifolia} \) (i.e., a species showing UI with \( N. \text{alata} \) that conforms to the SI \( \times \) SC rule). \( N. \text{plumbaginifolia} \) remains compatible with either \( N. \text{tabacum} \) (Figure 3A) or \( N. \text{plumbaginifolia} \) (Figure 3B) expressing \( S_A^2 \) RNase, and with \( N. \text{tabacum} \) expressing \( S_{C10} \) RNase (Figure 3B). However, Figure 4A shows that expression of \( S_A^2 \) RNase in \((N. \text{plumbaginifolia} \times \text{SC N. alata})\) hybrids leads to rejection of \( N. \text{plumbaginifolia} \) pollen. The data in Figure 4A show seven second generation transgenic plants expressing \( S_A^2 \) RNase with a change in pollination phenotype. These plants represent three independent transmants and two different constructs. Hybrids expressing \( S_A^2 \) RNase showed rejection of \( N. \text{plumbaginifolia} \) pollen in all of 25 trial pollinations. Untransformed hybrids are compatible with \( N. \text{plumbaginifolia} \) pollen (Figure 4A). There is a strict correlation between plants that show \( S \) allele–specific pollen rejection and those that show the ability to reject \( N. \text{plumbaginifolia} \) pollen.

\( N. \text{plumbaginifolia} \) pollen is usually rejected by either SI \( N. \text{alata} \) \( S_A^2S_A^2 \) or \((N. \text{plumbaginifolia} \times \text{SI N. alata} \) \( S_A^2S_A^2 \)) hybrids (zero of four compatible pollinations in Figure 1, and zero of seven in Figure 4B, respectively). To test whether S RNase is specifically required for rejection of \( N. \text{plumbaginifolia} \) pollen, \( S_A^2 \) RNase expression was suppressed with antisense \( S_A^2 \) RNase constructs. The data in Figure 4B show that antisense suppression of \( S_A^2 \) RNase in \((N. \text{plumbaginifolia} \times \text{SI l. alata} \) \( S_A^2S_A^2 \)) hybrids eliminates the ability to reject both \((N. \text{plumbaginifolia} \) pollen and \( N. \text{alata} \) \( S_A^2 \)) pollen. Six second generation plants, each derived from an independent transformant, showed low stylar RNase activities and low immunostaining with the anti-\( S_A^2 \) RNase antibody, and they consistently accepted pollen from \( N. \text{plumbaginifolia} \) and \( N. \text{alata} \) \( S_A^2S_A^2 \) (Figure 4B). Unsuppressed plants showed no change in phenotype. Again, there is a direct correlation between rejection of \( N. \text{plumbaginifolia} \) pollen and \( S \) allele–specific pollen rejection.

S RNase–Dependent Rejection of \( N. \text{tabacum} \) and \( N. \text{glutinosa} \) Pollen

As discussed above, interspecific compatibility relationships between \( N. \text{alata} \) and the SC species \( N. \text{glutinosa} \) and \( N. \text{tabacum} \) do not conform to the SI \( \times \) SC rule. Pollen from these species is rejected by an accession of \( N. \text{alata} \) that does not express an S RNase; this therefore represents an S RNase–independent pollen rejection mechanism. However, the existence of such a mechanism does not preclude the involvement of S locus products in rejecting pollen from these species. In contrast to the results described above for \( S \) allele–specific pollen rejection and rejection of pollen from \( N. \text{plumbaginifolia} \), the results in Figures 3A to 3C show that expression of S RNase in purely SC genetic backgrounds can cause rejection of pollen from \( N. \text{tabacum} \) and \( N. \text{glutinosa} \). Figure 3A shows that the six \( N. \text{tabacum} \) plants with the highest \( S_A^2 \) RNase expression levels (i.e., stylar RNase activity and reaction to the anti-\( S_A^2 \) RNase antibody comparable to \( N. \text{alata} \) \( S_A^2S_A^2 \)) rejected \( N. \text{tabacum} \) pollen (a total of three of 30 compatible pollinations; Figure 3A). Five of these six plants were also tested with \( N. \text{glutinosa} \) pollen, and the results again showed consistent rejection (a total of zero of 16 compatible pollinations, excluding a plant that was only tested once; Figure 3A). Two plants with a lower level of \( S_A^2 \) RNase expression showed rejection of \( N. \text{glutinosa} \) pollen (zero of four and zero of one compatible pollinations; Figure 3A) but not \( N. \text{tabacum} \) pollen (three of three and three of three compatible pollinations; Figure 3A). Untransformed controls were compatible with both \( N. \text{tabacum} \) and \( N. \text{glutinosa} \). Thus, \( S_A^2 \) RNase in \( N. \text{tabacum} \) causes a change in phenotype after pollination with \( N. \text{tabacum} \) or \( N. \text{glutinosa} \) but no change in phenotype after pollination with \( N. \text{plumbaginifolia} \) or \( N. \text{alata} \) \( S_A^2S_A^2 \). Lower levels of \( S_A^2 \) RNase expression were needed to cause rejection of \( N. \text{glutinosa} \) pollen than of \( N. \text{tabacum} \) pollen.

The data in Figure 3B show that this effect was not restricted to \( S_A^2 \) RNase. Transgenic \( N. \text{tabacum} \) plants expressing \( S_{C10} \) RNase also failed to show either \( S \) allele–specific pollen rejection or interspecific \( N. \text{plumbaginifolia} \) pollen rejection but gained the ability to reject pollen from \( N. \text{tabacum} \) and \( N. \text{glutinosa} \) (Figure 3B). Five independent primary transformants expressing \( S_{C10} \) RNase showed this effect; they rejected pollen from \( N. \text{tabacum} \) and \( N. \text{glutinosa} \) in each of at least three attempts. Thus, a change in pollination phenotype can be induced with either \( S_A^2 \) RNase or \( S_{C10} \) RNase.

The results in Figure 3C indicate that \( S_A^2 \) RNase can also cause rejection of \( N. \text{tabacum} \) pollen when expressed in the \( N. \text{plumbaginifolia} \) genetic background. Three second generation plants containing the pSA2S13 construct consistently rejected \( N. \text{tabacum} \) pollen (i.e., one of 13 compatible pollinations). Plants with lower \( S_A^2 \) RNase expression levels exhibited rejection in some but not all pollinations. Although pistils of untransformed \( N. \text{plumbaginifolia} \) plants support the growth of \( N. \text{glutinosa} \) pollen tubes, seed set did not occur and pollen tube morphology was somewhat abnormal (Figure 1). Therefore, only a limited number of the transgenic \( N. \text{plumbaginifolia} \) plants were tested for compatibility with \( N. \text{glutinosa} \). In these plants, inhibition of pollen tube growth occurred in the upper pistil, suggesting that \( N. \text{glutinosa} \) pollen tubes are also susceptible to S RNase–dependent inhibition in the \( N. \text{plumbaginifolia} \) background (data not shown).
Interspecific Pollen Rejection and Pollen Tube Morphology

To characterize further the S RNase–dependent pollen rejection phenotypes in these transgenic plants, styles were harvested 48 hr after pollination and stained with decolorized aniline blue. We have shown previously that S allele-specific pollen rejection in transgenic plants resembles the SI response in *N. alata* (Murfett et al., 1994, 1995).

Figure 5 shows the morphology of *N. plumbaginifolia* pollen tubes in transformed and control (*N. plumbaginifolia* x *N. alata*) hybrids. In untransformed (*N. plumbaginifolia* x SC *N. alata*) hybrids, *N. plumbaginifolia* pollen tubes have typical compatible morphology (de Nettancourt, 1977), penetrating the full length of the style, with thin walls and regularly spaced callose plugs (Figure 5A, left). However, when S<sub>A2</sub> RNase is expressed in these hybrids, inhibition of *N. plumbaginifolia* pollen occurs in the stigma soon after germination (Figure 5A, center). The inhibited pollen tubes show a heavy deposition of callose, particularly near the tube tip. Penetration into the style is extremely rare (Figure 5A, right), and no tubes grow beyond the top portion of the style. This stigmatic inhibition resembles inhibition in untransformed (*N. plumbaginifolia* x SI *N. alata S<sub>A2</sub>S<sub>A2</sub>) hybrids (Figure 5B, left and center) and is

![Figure 5](image-url)

**Figure 5.** Morphology of *N. plumbaginifolia* Pollen Tubes in Pistils of Transformed and Untransformed (*N. plumbaginifolia* x *N. alata*) Hybrids. 

(A) (*N. plumbaginifolia* x SC *N. alata*) hybrids. At left are typical compatible pollen tubes observed in the style transmitting tract of untransformed plants. At center is shown that most *N. plumbaginifolia* pollen tubes are arrested soon after germination in the stigmatic exudate of a (*N. plumbaginifolia* x SC *N. alata*) hybrid expressing S<sub>A2</sub> RNase from the pSA2S13 construct. Pollen grains and tubes fluoresce brightly against a background of weakly fluorescent stigmatic material. No callose plugs are evident. At right, among many squashes examined, a single thick-walled pollen tube was seen penetrating the transmitting tract. The pollen tube was not observed lower in the style. A vascular bundle (pale green) and transmitting tract cells (blue) are also visible.

(B) (*N. plumbaginifolia* x SI *N. alata S<sub>A2</sub>S<sub>A2</sub>) hybrids. At left are arrested *N. plumbaginifolia* pollen tubes and ungerminated pollen grains on the stigma surface of an untransformed plant. At center, a region of the style just below the stigma is shown. The blue transmitting tract is devoid of pollen tubes. A fragment of epidermal tissue is visible at top. At right are compatible *N. plumbaginifolia* pollen tubes growing in the transmitting tract of a (*N. plumbaginifolia* x SI *N. alata S<sub>A2</sub>S<sub>A2</sub>) hybrid in which S<sub>A2</sub> RNase expression is suppressed by the pBAGA antisense construct.
A *N. tabacum* pollen in pistils of *N. tabacum*

![Image of pollen tubes in *N. tabacum* pistils](Figure 6A)

(A) *N. tabacum* pistils. At left are a large number of *N. tabacum* pollen tubes visible at the base of the style in an untransformed plant. The brightly fluorescing pollen tubes have thin cell walls and regularly spaced callose plugs, which is typical of compatible tubes. A few epidermal cells are visible at the upper right. The morphology of *N. tabacum* pollen tubes arrested in the upper style of an *N. tabacum* plant expressing S\(^{A2}\) RNase from the pSA29617 construct is shown at center. Pollen tubes show thickened walls, uneven growth, and swollen tips. A portion of the lower style, devoid of pollen tubes, is shown at right.

B *N. tabacum* pollen in pistils of *N. plumbaginifolia*

![Image of pollen tubes in *N. plumbaginifolia* pistils](Figure 6B)

(B) *N. plumbaginifolia* pistils. At left are healthy *N. tabacum* pollen tubes observed near the base of the style of an untransformed *N. plumbaginifolia* plant. *N. tabacum* pollen tubes arrested in the upper style of an *N. plumbaginifolia* plant expressing S\(^{A2}\) RNase from the pSA2S13 construct are shown at center. A portion of the lower style, showing absence of pollen tubes, is shown at right.

typical of interspecific pollen rejection in *Nicotiana* (Pandey, 1979). The inhibition of pollen tube growth is relieved by antisense suppression of S\(^{A2}\) RNase expression in the transgenic (*N. plumbaginifolia* × SI *N. alata* S\(^{A2}\)S\(^{A2}\)) hybrids (Figure 5B, right), confirming that S\(^{A2}\) RNase is required.

Figure 6A shows the morphology of *N. tabacum* pollen tubes in styles of untransformed and transformed *N. tabacum* plants. Again, in untransformed plants, the pollen tubes have typical compatible morphologies with relatively thin walls and numerous callose plugs. Many tubes have penetrated to the bottom of the style by ~48 hr after pollination. However, in plants expressing S\(^{A2}\) RNase, *N. tabacum* pollen tubes typically penetrate <1 cm of the style after 48 hr. These inhibited pollen tubes show a heavy deposition of callose in the tube wall and few callose plugs. Similar alterations in pollen tube morphology are associated with inhibition of pollen tube growth due to SI (de Nettancourt, 1977; Murfett et al., 1994). Figure 6B shows that the morphology of inhibited *N. tabacum* pollen tubes in transgenic *N. plumbaginifolia* plants is similar to that observed in transgenic *N. tabacum* plants (Figure 6A versus Figure 6B).

Figure 7 shows comparable results for inhibition of *N. glutinosa* pollen in transgenic *N. tabacum* plants expressing S\(^{C10}\) RNase. In transgenic *N. tabacum* plants that express S\(^{C10}\)
**N. glutinosa** pollen in pistils of transformed *N. tabacum*

Figure 7. Morphology and Location of *N. glutinosa* Pollen Tube Inhibition in Transgenic *N. tabacum* Plants Expressing S<sub>C10</sub>-RNase.

(Upper left) Compatible *N. glutinosa* pollen tubes. These pollen tubes reached the base of the style within 48 hr and show a typical compatible morphology.

(Upper right) No *N. glutinosa* pollen tubes are visible at the base of the style in a plant expressing S<sub>C10</sub>-RNase. Brightly stained epidermal cells are visible at the top of this field.

(Lower left) An inhibited *N. glutinosa* pollen tube photographed in the transition region between the stigma and the stylar transmitting tract. The uneven walls and swollen tip are typical of incompatible pollen tubes.

(Lower right) Inhibition of *N. glutinosa* pollen tube growth soon after germination on the stigma surface. The pollen tubes in this region are unusually distorted, and the bright staining is due to heavy deposition of callose. The pollen tubes are highly contorted. In *N. tabacum* plants expressing S<sub>C10</sub> RNase, few *N. glutinosa* pollen tubes are observed below the stigma surface 48 hr after pollination.

RNase at high levels, rejection of *N. glutinosa* pollen usually occurs in the stigma or in the transition zone between the stigma and the style. Figure 7 shows an unusually large number of poorly formed pollen tubes on the stigma surface. The few *N. glutinosa* pollen tubes that penetrate to the upper portion of the transmitting tract have swollen tips and heavy callose deposition similar to the phenotype associated with SI pollen rejection.

**DISCUSSION**

The relationship between SI and unilateral interspecific incompatibility has been controversial for many years, in spite of considerable genetic evidence implicating the S locus in several interspecific incompatibility systems. There are two key arguments against the involvement of the S locus in UI. First, in many cases, UI crossing relationships are retained in SC accessions of normally SI species. Second, the morphologies of pollen tubes inhibited by UI are often different from those of pollen tubes inhibited by SI. These difficulties led to formulation of the incongruity hypothesis, stating that interspecific pollen rejection is a multigenic trait in which the S locus plays no special role (Hogenboom, 1975, 1984; Mutschler and Leidl, 1994).

We used cloned S RNase genes from *N. alata* and plant transformation to address the question of whether S locus products can cause rejection of pollen from SC species. The S RNase expression system and the antisense constructs used to suppress S<sub>A2</sub> RNase have been described previously (Murfett et al., 1994, 1995). The results in Figures 3 and 4 show that S RNase expression was altered in the transgenic plants. It is known that non-S RNases are expressed in the pistils of solanaceous plants (McClure et al., 1989; Lee and Kao, 1992). However, the total activity contributed by these enzymes is relatively low, and therefore, expression of S RNase in the transgenic plants was correlated with an increase in stylar RNase specific activity compared with untransformed controls (Figures 3A to 3C, and 4A). Antisense-transformed plants showed a decrease in stylar RNase specific activity (Figure 4B). In previous studies, we used RNA gel blot analysis and S<sub>A2</sub> RNase–specific probes to confirm that altered stylar RNase activity is due to expression of the transgene. An increase in S<sub>A2</sub> RNase transcript was associated with increased style RNase activity in plants transformed with an S<sub>A2</sub> RNase expression construct (Murfett et al., 1994), and a decrease in S<sub>A2</sub> RNase transcript was associated with decreased style RNase activity in antisense S<sub>A2</sub> RNase–transformed plants (Murfett et al., 1995). In this study, S RNase transgene expression levels were monitored by both RNase specific activity and immunostaining with anti-S<sub>A2</sub> and anti-S<sub>C10</sub> RNase-specific antibodies. These antibodies did not cross-react with non-S RNases present in the styles of untransformed control plants (Figures 3 and 4). Transformed plants showing increased stylar RNase specific activity also showed a positive reaction with the antibodies, and antisense-suppressed plants showed a decreased reaction with the antibody. This correlation held in multiple independent transformants and in several genetic backgrounds transformed with a variety of constructs. Therefore, the changes in pollination phenotype described below can be attributed to changes in S RNase expression.
Whereas \textit{S}_{A2} RNase expressed in the transgenic plants comigrated with the protein expressed in \textit{N. alata}, \textit{S}_{C10} RNase showed aberrant migration (Figures 3A, 3C, and 4A versus Figure 3B). This aberrant migration was not observed when \textit{S}_{C10} RNase was expressed in \textit{N. plumaginifolia} (B.A. McClure, unpublished data). Although the reason for this aberrant migration is not known, the pattern is similar to that observed for differences in glycosylation. Multiple glycoforms of \textit{Brassica} \textit{S} locus–specific glycoproteins and \textit{S} locus–related proteins exist (Nasrallah et al., 1985; Umbach et al., 1990), although the functional significance of these isoforms is not known. The \textit{S}13 \textit{S} locus–specific glycoprotein protein was also heterogeneous when expressed in \textit{N. tabacum} (Moore and Nasrallah, 1990). \textit{S}2 RNase showed heterogeneity in SDS–polyacrylamide gels, when style extracts from \textit{N. alata} \textit{S}2\textit{S}2 were examined (Anderson et al., 1986). Detailed analysis of \textit{N. alata} \textit{S}2 RNase expressed in \textit{N. tabacum} revealed no differences in glycosylation between the protein expressed in transgenic plants and the protein isolated from \textit{N. alata} (Murfett et al., 1992). Moreover, it has been shown that the soxal activity is not required for \textit{S} allele–specific pollen rejection in \textit{P. inflata} (Karunananda et al., 1994). As discussed below, both \textit{S}_{A2} and \textit{S}_{C10} RNase were active in pollen rejection and had enzymatic activity, but only \textit{S}_{C10} RNase showed heterogeneity in SDS-PAGE. Thus, the significance of the heterogeneity of \textit{S}_{C10} RNase expressed in \textit{N. tabacum} is questionable because it does not appear to be correlated with a functional difference.

\hspace{1cm}

**S RNase-Dependent and S RNase-Independent Pollen Rejection Mechanisms**

In this study, we examined the relationship between SI and UI in three separate systems by comparing the pollination behavior (i.e., compatibility and pollen tube morphology) of untransformed plants with the behavior of plants transformed with a single cloned gene (i.e., \textit{S} RNase) from \textit{N. alata}. The results suggest a complex and diverse set of pollen rejection mechanisms. Pollen from SI \textit{N. plumaginifolia} is rejected by SI accesses of \textit{N. alata} but accepted by the SC accession cultivar Breakthrough (Figure 1). Thus, UI between \textit{N. plumaginifolia} and \textit{N. alata} conforms to the SI \times SC rule. However, Figure 1 shows that even though the SC accession of \textit{N. alata} does not express an \textit{S} RNase, it retains the ability to reject pollen from \textit{N. tabacum} and \textit{N. glutinosa}. Therefore, UI between these species and \textit{N. alata} provides examples of exceptions to the SI \times SC rule. These exceptions are similar to those used to argue against the involvement of the \textit{S} locus in UI (Hogenboom, 1975, 1984). Clearly, one or more \textit{S} RNase–independent pollen rejection mechanisms operate in \textit{N. alata}. The nature of \textit{S} RNase–independent pollen rejection and its relationship, if any, to intraspecific SI pollen rejection is not known. As discussed below, our results suggest that \textit{N. tabacum} and \textit{N. glutinosa} pollen are also susceptible to \textit{S} RNase–mediated rejection in SI \textit{N. alata}.

**Similarities between S Allele-Specific Pollen Rejection and Rejection of N. plumaginifolia Pollen**

We compared the pollination phenotypes of transgenic \textit{N. plumaginifolia} and \textit{N. tabacum} plants with transgenic (\textit{N. plumaginifolia} \times \textit{N. alata}) hybrids to address the question of whether \textit{S} RNase–mediated pollen rejection is dependent on genetic background. Interspecific hybrids have been used extensively in previous genetic studies of SI and interspecific incompatibility (McGuire and Rick, 1954; Martin, 1961, 1967; Al et al., 1991; Chetelat and DeVerna, 1991; Bernatzky et al., 1995). Although (\textit{N. plumaginifolia} \times \textit{N. alata}) hybrids do not set seed, pollination phenotypes are easily scored by ovary swelling and can be confirmed by examination of pollen tube morphology (Murfett et al., 1995). Examination of pollen tube morphology rather than seed set is a traditional approach to scoring incompatibility between species of \textit{Nicotiana} (Pandey, 1981).

By examining pollination behavior of transgenic \textit{N. plumaginifolia} and \textit{N. tabacum} plants expressing \textit{S} RNase, we showed that \textit{S} RNase alone is not sufficient for \textit{S} allele–specific pollen rejection or rejection of \textit{N. plumaginifolia} pollen in these purely SC genetic backgrounds (Figures 3A to 3C). Even plants expressing \textit{S}_{A2} RNase or \textit{S}_{C10} RNase at levels similar to those observed in SI \textit{N. alata} accept pollen from \textit{N. plumaginifolia}, \textit{N. alata} \textit{S}_{A2}\textit{S}_{A2}, and \textit{N. alata} \textit{S}_{C10}\textit{S}_{C10} with equal facility (Figures 3A to 3C). However, when \textit{S}_{A2} RNase is expressed in conjunction with other factors from the \textit{N. alata} genetic background, transgenic plants simultaneously gain the ability to reject \textit{N. plumaginifolia} pollen and perform the \textit{S} allele–specific pollen rejection characteristic of SI. This effect was observed when \textit{N. plumaginifolia} plants transformed with \textit{S}_{A2} RNase constructs were crossed with an SC accession of \textit{N. alata} that did not express an \textit{S} RNase, thus generating transgenic (\textit{N. plumaginifolia} \times SC \textit{N. alata}) hybrids (Figure 4A). This strategy avoids the difficulty of obtaining large numbers of \textit{N. alata} transformants (Ebert and Clarke, 1990).

Compared with untransformed controls, expression of \textit{S}_{A2} RNase alters the pollination phenotype of (\textit{N. plumaginifolia} \times SC \textit{N. alata}) hybrids in a highly specific manner. Transgenic hybrids expressing \textit{S}_{A2} RNase accept pollen from \textit{N. alata} \textit{S}_{C10}\textit{S}_{C10} but gain the ability to reject pollen from \textit{N. plumaginifolia} and \textit{N. alata} \textit{S}_{A2}\textit{S}_{A2} (Figure 4A). There is a strict correlation between \textit{S} allele–specific pollen rejection and rejection of \textit{N. plumaginifolia} pollen. By examining style squashes stained with decolorized aniline blue, we determined that \textit{S}_{A2} RNase–dependent rejection of \textit{N. plumaginifolia} pollen in the transgenic hybrids resembles a typical UI response (Figure 5A). Similar results have been obtained with \textit{S}_{C10} RNase. \textit{N. plumaginifolia} plants expressing \textit{S}_{C10} RNase fail to reject either \textit{N. plumaginifolia} pollen or \textit{N. alata} \textit{S}_{C10} pollen, but both types of pollen are rejected when \textit{S}_{C10} RNase is expressed in the (\textit{N. plumaginifolia} \times SC \textit{N. alata}) hybrid (B.A. McClure and B. Mou, unpublished data).

Antisense suppression experiments indicated that \textit{S} RNase expression is necessary for rejection of \textit{N. plumaginifolia}
pollen by *N. alata*. Untransformed (*N. plumbaginifolia* × SI *N. alata* SxA2Sx2) hybrids rejected *N. plumbaginifolia* pollen (Figure 4B). However, when *N. plumbaginifolia* transformed with antisense SxA2 RNase constructs was crossed with *N. alata* SxA2Sx2, the transgenic (*N. plumbaginifolia* × SI *N. alata* SxA2Sx2) hybrids showed suppression of stylar RNase activity (Figure 4B). In hybrids in which SxA2 RNase expression is strongly suppressed, the plants simultaneously lost the ability to reject pollen from both *N. plumbaginifolia* pollen and *N. alata* SxA2Sx2 (Figure 4B). These changes in pollination phenotype are mirrored in changes to typical compatible pollen tube morphologies (Figure 5B; Murfett et al., 1995).

We conclude that rejection of *N. plumbaginifolia* pollen is similar to S allele–specific pollen rejection. Both require high-level expression of S RNase and non-S RNase factors present in the *N. alata* genetic background. The dependence on non-S RNase factors is consistent with earlier studies, suggesting that multiple genes are involved in interspecific (Chetelat and DeVerna, 1991) and intraspecific pollen rejection (Ai et al., 1991; Bernatzky et al., 1995). However, it has never been clear whether these additional factors act in the pollen or the pistil. Because our experiments always involved pollination with untransformed testers, it is clear that the factor(s) active in our system acts in the pistil. Detailed genetic analysis has not been performed, but the results presented here suggest genetic dominance (i.e., the factor(s) functions in the [N. plumbaginifolia × SC N. alata] F1, hybrid). How the factor(s) modifies the function of S RNase is not known. SxA2 RNase expressed in *N. plumbaginifolia* and *N. tabacum* comigrated with SxA2 RNase from *N. alata* (Figures 3A and 3B), and proteins from both sources are active ribonucleases. This suggests, but does not prove, that there are no gross structural differences in the protein expressed in these genetic backgrounds. It is also uncertain whether the factor(s) required for style part function in SI and for interspecific rejection of *N. plumbaginifolia* pollen will prove to be identical.

The similarity between rejection of *N. plumbaginifolia* pollen and SI directly relates to the controversy over the relationship between interspecific and intraspecific pollen rejection. Our results provide clear evidence that the S locus is required for both SI and rejection of *N. plumbaginifolia* pollen. The interspecific pollen rejection mechanism described here is not unique to *N. plumbaginifolia*. We have preliminary evidence that rejection of pollen from SC *N. longiflora* shows the same dependence on high-level S RNase expression and non-S RNase factor(s) (B.A. McClure and J. Lyon, unpublished data). Both *N. plumbaginifolia* and *N. longiflora* follow the SI × SC rule in that their pollen is rejected by SI accessions of *N. alata* and accepted by the SC accession (i.e., cultivar Breakthrough).

If the SI pollen-rejection mechanism were the only pathway for rejection of interspecific pollen by SI plants, then loss of stylar SI components would always be accompanied by loss of the ability to reject interspecific pollen. Many interspecific pollen incompatibility relationships (i.e., such as rejection of *N. plumbaginifolia* pollen by *N. alata*) are consistent with this prediction (Lewis and Crowe, 1958; de Nettancourt, 1977). Our speculation is that in interspecific incompatibility systems that follow the SI × SC rule, the pollen-rejection mechanisms will closely resemble the SI pollen-rejection mechanism. However, there are many exceptions to the rule, such as rejection of *N. tabacum* and *N. glutinosa* pollen by both SI and SC accessions of *N. alata*; these exceptions are discussed below.

### S RNase-Dependent Rejection of *N. glutinosa* and *N. tabacum* Pollen

Unlike the results described above, the results in Figures 3A to 3C show that introducing a single cloned S RNase gene into *N. tabacum* or *N. plumbaginifolia* causes a change in phenotype when the plants are tested with pollen from *N. glutinosa* or *N. tabacum*. When either SxA2 or Sx2 RNase is expressed above a threshold level, transgenic *N. tabacum* plants gain the ability to reject pollen from *N. glutinosa* and *N. tabacum* (Figures 3A and 3B). Plants with lower SxA2 RNase expression levels appear to reject *N. glutinosa* pollen but not *N. tabacum* pollen, suggesting that *N. glutinosa* is more sensitive to this pollen-rejection mechanism. Figure 3C shows similar results in a second SC genetic background, *N. plumbaginifolia*. However, because *N. glutinosa* pollen tubes grow somewhat abnormally in *N. plumbaginifolia* pistils, results are presented only for *N. tabacum* pollinations.

We conclude that the S locus is implicated in rejecting pollen from *N. tabacum* and *N. glutinosa* because rejection can be induced with either of two S RNases and in two genetic backgrounds. It appears that *N. alata* has redundant mechanisms for rejecting pollen from these species. In vivo, S RNase–dependent and S RNase–independent pollen-rejection pathways may both contribute to UI between these two SC species and SI *N. alata*. However, this type of S RNase–dependent pollen rejection is different from the mechanism used to reject *N. plumbaginifolia* pollen and the SI mechanism, because other genetic factors from *N. alata* are not required.

### Morphology and Site of Pollen Tube Inhibition

Above, we showed that rejection of *N. plumbaginifolia* pollen is similar to S allele–specific pollen rejection based on similar requirements for both non-S RNase factors and S RNase. The results in Figure 5 show that in untransformed (*N. plum- baginifolia* × SI *N. alata* SxA2Sx2) hybrids, inhibition of *N. plumbaginifolia* pollen tubes occurs in the stigma soon after tube emergence (Figure 5B). Stigmatic inhibition was also observed in (*N. plumbaginifolia* × SC *N. alata*) hybrids expressing SxA2 RNase (Figure 5A). In contrast, S allele–specific pollen tube inhibition in SI normally occurs later, after the pollen tubes have penetrated into the upper portion of the style (de Nettancourt, 1977; Murfett et al., 1994, 1995). Thus, in spite
of their similar genetic mechanisms, the morphologies and sites of pollen tube inhibition are different. Figure 6 shows that inhibition of N. tabacum pollen tubes occurs in the style of transgenic N. tabacum and N. plumbaginifolia plants expressing S\(_{32}\) RNase. In this respect, the morphology of the inhibited N. tabacum pollen tubes resembles the morphology of pollen tubes inhibited through the SI mechanism. However, these two pollen-rejection mechanisms are distinct because they do not require the same accessory genetic factors. Although inhibition of N. glutinosa pollen tubes appears to be mechanistically similar to N. tabacum, the inhibition reaction occurs much earlier (Figure 6 versus Figure 7). In SC N. alata, inhibition of N. tabacum pollen tube growth (i.e., S RNase–independent inhibition) occurred somewhat later than in SI N. alata but earlier than in transgenic plants expressing S RNase (B.A. McClure, unpublished data). This result is consistent with concerted action of S RNase–dependent and S RNase–independent pollen-rejection pathways. However, our overall conclusion is that the morphology and site of pollen tube inhibition are not good indicators of the mechanism of pollen tube inhibition.

**Complex Mechanisms for Interspecific Pollen Rejection**

Our results provide strong evidence for the involvement of the S locus in rejecting pollen from SC species in the genus *Nicotiana*. However, interspecific pollen rejection is complex. There appear to be multiple mechanisms, and these may show overlap in the gene products involved and redundancy in the specificity of rejection. Preliminary results suggest that besides the three SC species examined here, SC N. sylvestris and N. longiflora are also sensitive to S RNase–dependent pollen-rejection mechanisms (data not shown). Rejection of N. sylvestris pollen by N. alata appears to be similar to rejection of N. tabacum and N. glutinosa pollen. Rejection of N. longiflora pollen appears to be similar to rejection of N. plumbaginifolia pollen and to the SI pollen-rejection mechanism.

The observation that N. alata has redundant mechanisms for interspecific pollen rejection may explain why some UI systems follow the SI × SC rule and others do not. There are at least two types of mutations that would result in loss of SI function in the style. SC could result from alteration of a non–S RNase factor, as has been demonstrated in petunia (Ai et al., 1991). Alternatively, S RNase itself could be absent or defective, as in SC N. alata cv Breakthrough. In either case, such plants would lose the ability to reject pollen from SC species, such as N. plumbaginifolia, in which the interspecific pollen-rejection mechanism closely resembles S allele–specific pollen rejection. This is the behavior expected from the SI × SC rule. However, rejection of pollen from such species as N. glutinosa and N. tabacum that are inhibited by an S RNase–independent mechanism could still occur in either type of SC mutant. Thus, the presence of redundant pollen-rejection mechanisms implies that breakdown of SI does not always lead to loss of interspecific pollen rejection. Further complexities arise from the mechanistic overlap between different interspecific pollen-rejection mechanisms. For example, S RNase is implicated in rejecting pollen from all three SC species used in this study. However, at least two different S RNase–dependent mechanisms can be distinguished.

Pandey has described a hierarchy of interspecific cross-compatibility relationships in the genus *Nicotiana* (Pandey, 1969, 1973, 1979, 1981). The species can be classified into interspecific compatibility groups based on crossing behavior when used as pollen or pistil parents. For example, species such as N. tabacum, which accept pollen from many other species, show very restricted cross-compatibility when used as pollen parent. Conversely, species such as N. alata, which reject pollen from many species, are able to pollinate many other species. Thus, an inverse relationship is observed between behavior as pollen or pistil parent (Pandey, 1981). Pandey's observations could be explained by a series of overlapping pollen-rejection mechanisms such as those described here. For example, pollen from N. plumbaginifolia and N. alata is not inhibited by the S RNase–independent pollen-rejection pathways described for N. glutinosa and N. tabacum. Perhaps some resistance factors are expressed in the pollen of these species to overcome this inhibitory mechanism. Species such as N. tabacum and N. glutinosa, which do not express a pollen factor for resistance to the S RNase–independent pathway, would be unable to pollinate as many species as those species that do express such a factor. A series of such inhibitory factors in the style and resistance factors in pollen might comprise what Pandey (1981) referred to as matching elements of the “S gene complex.” However, we know of no evidence for genetic linkage between the S locus and factors affecting S RNase–independent pollen-rejection pathways, as was proposed by Pandey (1981). To characterize further these new pollen-rejection pathways, we must identify genes other than S RNase that are involved in controlling interspecific pollination.

For the interspecific pollination systems we have examined, we interpret the results as suggesting that interspecific pollen rejection is an active process. Our interpretation contrasts with the incongruity hypothesis of Hogenboom (1975, 1984). This hypothesis suggests that failure of interspecific pollination is best described as incongruity resulting from evolutionary divergence between species. The incongruity model emphasizes the extensive coevolution of the pollen and pistil, and the pressure to maintain productive interactions between them. Incongruity is not regarded as active rejection per se, as occurs in SI. Instead, it could result from evolutionary divergence of any gene that contributes to the productive interaction between the pollen and pistil of a single species. It is likely that many cases of interspecific incompatibility, particularly between distantly related species, result from incongruity. The incongruity concept might also be useful in explaining the factors that prevent zygote development and seed set in some interspecific crosses. However, the S locus was implicated in each example of interspecific pollen rejection that we examined.
METHODS

Plant Materials

Nicotiana glutinosa (inventory number TW58; accession number 24), N. plumbaginifolia (inventory number TW107; accession number 43B), and N. tabacum cv. Preaux (inventory number TI 1347) were obtained from the U.S. Tobacco Germplasm Collection (Crops Research Laboratory, Oxford, NC). A self-incompatible (SI) N. alata plant designated S\textsubscript{A2}S\textsubscript{A2} was force self-pollinated to generate the homozygous genotypes S\textsubscript{A2}S\textsubscript{A2} and S\textsubscript{C0}S\textsubscript{C0} (Murfett et al., 1994). Self-compatible (SC) N. alata cv. Breakthrough was obtained from Thompson and Morgan (Jackson, NJ).

S RNase Constructs and Plant Transformation

The S\textsubscript{A2} RNase expression construct pSA29617 has been described previously (Murfett et al., 1994). The S\textsubscript{SC} RNase expression construct pSC109617 contains the same ChiP promoter, S\textsubscript{SC} RNase secretory sequence, and S\textsubscript{A2} gene 3' untranslated sequences as pSA29617. However, in pSC109617, sequences encoding the mature S\textsubscript{SC} RNase protein replace the corresponding S\textsubscript{A2} RNase gene sequences. This construct provides high-level expression of S\textsubscript{SC} RNase in transgenic plants. In an appropriate genetic background, this construct causes transformed plants to specifically reject S\textsubscript{SC} pollen (B. Beecher, unpublished data). The antisense constructs have also been described previously (Murfett et al., 1995). Briefly, an S\textsubscript{A2} RNase cDNA clone was obtained from N. alata S\textsubscript{A2}S\textsubscript{A2}. The sequence contains an EcoRI linker just 5' of the initiator ATG and a unique BamHI site 50 bp from the 5' end. The 550-bp EcoRI-BamHI fragment was used to generate antisense constructs by using either the cauliflower mosaic virus 35S promoter with duplicated enhancer (Kay et al., 1987) from pAGUS1 (Skuzasaki et al., 1990) (pBAGA) or the tomato ChiP promoter (Gasser et al., 1993) (pHAKA). Both constructs include the nopaline synthase termination region (Sevan et al., 1983). Sense and antisense constructs were transferred to pBIN19 (Bevan, 1984) and mobilized into Agrobacterium tumefaciens LBA4404 for plant transformation, essentially as described by Murfett et al. (1992). N. tabacum and N. plumbaginifolia plants were transformed by the leaf disc method (Horsch et al., 1985), as described by Murfett et al. (1992). For plants transformed with S\textsubscript{A2} RNase expression constructs (pSA29617 and pSA2S13) and S\textsubscript{A2} RNase antisense constructs (pHAKA and pBAGA), primary N. tabacum and N. plumbaginifolia transformants were selfed or outcrossed to generate second generation transformed plants for pollination analysis. In the case of pSC109617, primary N. tabacum transformants were analyzed.

Protein Analysis

For the analysis of style proteins in Figure 1, mature styles were weighed, homogenized in SDS loading buffer (Laemmli, 1970), and boiled. Extract equivalent to 1.5 mg of fresh weight was separated by SDS-PAGE (15% T/0.8% C), and proteins were stained with Coomassie Brilliant Blue R 250. For immunostaining of S\textsubscript{A2} and S\textsubscript{SC} RNase, 20 μg of style protein was separated by SDS-PAGE in a 10% Tris-Tricine gel (Schagger and von Jagow, 1987). Proteins were electroblotted to nitrocellulose and detected either with an S\textsubscript{A2} RNase-specific rabbit polyclonal IgG preparation or with a mouse monoclonal antibody raised against deglycosylated S\textsubscript{SC} RNase. After treatment with an alkaline phosphatase-conjugated secondary antibody, immune complexes were detected with nitro blue tetrazolium/5-bromo-4-chloro-3-indoly phosphate (Harlow and Lane, 1988). Ribonuclease specific activities of style extracts were determined in duplicate, as described by McClure et al. (1989).

Pollination Phenotypes

Flowers were emasculated before floral maturity. Test pollinations were performed 2 days after the flowers opened by using pollen from untransformed N. glutinosa, N. tabacum, N. plumbaginifolia, SC N. alata, or SI N. alata genotypes S\textsubscript{A2}S\textsubscript{A2} or S\textsubscript{C0}S\textsubscript{C0}. Compatibility was scored as described by Pandey (1981). In cases in which fruit set did not occur, style squashes were used to determine whether failure to set seed was due to pollen rejection or seed abortion. The style squashes were prepared from pistils harvested 48 hr after pollination and stained with decolorized aniline blue (Kho and Baer, 1968). For example, in Figure 1, N. glutinosa fails to set seed with all species tested; yet, these pollinations are scored as compatible (Pandey, 1981) because microscopic examination revealed numerous healthy pollen tubes reaching the base of the style 48 hr after pollination. The (N. plumbaginifolia × SC N. alata) and (N. plumbaginifolia × SI N. alata S\textsubscript{A2}S\textsubscript{A2}) hybrids are sterile. For these hybrids, compatible and incompatible pollinations were scored by ovary swelling. Ovaries were weighed 5 to 7 days after pollination. After compatible pollinations, the ovaries swelled to approximately double their original size and weight; after incompatible pollinations, little or no swelling occurred (Murfett et al., 1995). Pollination phenotypes were confirmed by microscopic examination of pollen tube morphology. Pistils were harvested 48 hr after pollination, stained with decolorized aniline blue (Kho and Baer, 1968), and viewed by epifluorescence. From two to eight pollinations were examined for each type of cross that involved different species or hybrids. The photographs shown in Figures 5 to 7 are representative.

ACKNOWLEDGMENTS

We thank Dr. Charles S. Gasser (University of California, Davis) for the ChiP promoter and Dr. June B. Naareliah (Cornell University, Ithaca, NY) for the SLG\textsubscript{A2} gene promoter. We also thank Drs. James Birchler and Timothy P. Holtsford (University of Missouri—Columbia) for helpful discussions. This work was supported by the University of Missouri Research Board, the Food for the 21st Century Program, and National Science Foundation Grant No. 93-16152.

REFERENCES


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.

J. Murfett, T. J. Strabala, D. M. Zurek, B. Mou, B. Beecher and B. A. McClure

*Plant Cell* 1996;8:943-958

DOI 10.1105/tpc.8.6.943

This information is current as of August 5, 2017