A small asparagine-rich protein required for S-allele-specific pollen rejection in *Nicotiana*

Bruce McClure*, Beiquan Mou†, Stefano Canevascini‡, and Robert Bernatzky§

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

Edited by C. S. Levings III, North Carolina State University, Raleigh, and approved August 17, 1999 (received for review April 30, 1999)

Although S-locus RNases (S-RNases) determine the specificity of pollen rejection in self-incompatible (SI) solanaceous plants, they alone are not sufficient to cause S-allele-specific pollen rejection. To identify non-S-RNase sequences that are required for pollen rejection, a *Nicotiana alata* cDNA library was screened by differential hybridization. One clone, designated HT, hybridized strongly to RNA from *N. alata* styles but not to RNA from *Nicotiana plumbaginifolia*, a species known to lack one or more factors necessary for S-allele-specific pollen rejection. Sequence analysis revealed a 101-residue ORF including a putative secretion signal and an asparagine-rich domain near the C terminus. RNA blot analysis showed that the HT-transcript accumulates in the stigma and style before anthesis. The timing of HT-expression lags slightly behind S_{C10}-RNase in SI *N. alata* $S_{C10}S_{C10}$ and is well correlated with the onset of S-allele-specific pollen rejection in the style. An antisense-HT construct was prepared to test for a role in pollen rejection. Transformed (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrids with reduced levels of HT-protein continued to express S_{C10}-RNase but failed to reject S_{C10}-pollen. Control hybrids expressing both S_{C10}-RNase and HT-protein showed a normal S-allele-specific pollen rejection response. We conclude that HT-protein is directly implicated in pollen rejection.

Self-incompatibility (SI) systems are the best understood pollen rejection mechanisms. Many plants have SI systems in which the specificity of pollen rejection is controlled by a single locus, referred to as the S-locus (1, 2). In the Solanaceae, pollen is rejected when the single S-allele in a haploid pollen tube matches either of the two S-alleles in the diploid pistil. In the pistil, the products of the S-locus are secreted ribonucleases called S-RNases (3). Each S-allele encodes a different S-RNase that contains the specificity determinants for S-allele-specific recognition by pollen. RNase activity is required for pollen rejection (4), and it is generally accepted that S-RNases act as S-allele-specific cytotoxins that inhibit growth of pollen bearing a matching S-allele (5, 6). The nature of the specificity determinant in pollen is not known, but it is distinct from S-RNase.

By definition, the S-locus encodes the determinants of allelic specificity. However, other loci are also required for pollen rejection. In SI *Brassica*, an aquaporin gene has been shown to be required for pollen rejection (7). In *Brassica* and in *Papaver*, gene products that bind to S-proteins have been identified, but it is not yet known whether they play a direct role in pollen rejection (8–10). In early studies in the Solanaceae, East demonstrated a requirement for multiple loci in SI and also suggested that such factors may interact differently with different S-alleles (11). Recently, Bernatzky *et al.* (12) generated self-compatible (SC) *Lycopersicon esculentum* lines containing SI *Lycopersicon hirsutum* chromosome fragments bearing the S-locus, providing further genetic evidence that the S-RNases alone are not sufficient for SI. Similarly, an S-allele from SC *Petunia hybrida* cv. Strawberry Daddy was shown to be functional when crossed into SI *Petunia inflata*, suggesting that a factor from the SI background could complement a factor missing in cv. Strawberry Daddy (13). By using plant transformation, we showed that, when S-RNase is expressed in transgenic SC *Nicotiana plumbaginifolia*, it does not cause S-allele-specific pollen rejection. However, when it is expressed in (*N. plumbaginifolia* × SC *Nicotiana alata*) hybrids, both S-allele-specific pollen rejection and a type of interspecific pollen rejection occur normally (14). Thus, when present in *trans*, factors from the SC *N. alata* background allow the S-RNase transgene to function in SI and interspecific pollen rejection. The identities and functions of these factors are not known.

We used a differential cDNA-cloning approach to identify putative non-S-RNase factors required for S-allele-specific pollen rejection in the style. Here, we report cloning a cDNA encoding a small asparagine-rich protein expressed late in style development. Antisense transformed (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrids showing reduced expression of this protein accept *N. alata* S_{C10} pollen even though they still express S_{C10}-RNase.

Materials and Methods

Plant Materials and Transformation. *Nicotiana longiflora* (inventory no. TW79, accession no. 30A) was obtained from the U.S. Tobacco Germplasm Collection (Crops Research Laboratory, Oxford, NC). All other plant materials have been previously described (14–16). For the antisense experiments, transgenic *N. plumbaginifolia* plants were generated by *Agrobacterium*-mediated transformation of leaf explants (17).

cDNA Cloning. Polyadenylated RNA was prepared from mature *N. plumbaginifolia* and SC *N. alata* cv. Breakthrough styles. cDNA libraries of stylar SC *N. alata* sequences were then prepared in λ-gt-10 (Promega) and λ-ZIPLOX (Life Technologies, Grand Island, NY) by following recommended procedures. The λ-gt-10 library was screened by differential hybridization with ^{32}P-labeled cDNA prepared from *N. plumbaginifolia* and SC *N. alata* style RNA. DNA sequencing and RNA blot analyses showed that the HT cDNAs were incomplete. The λ-ZIPLOX library was therefore screened to obtain a full-length clone.

Antisense Experiments. The HT-cDNA was engineered to create a *Sac*I site 27 bp upstream of the initiation codon and a *Bam*HI site 146 bp past the terminator by PCR with the synthetic oligonucleotides GCTTGGATCCTTATTACAAACAAAGTGGAAATTAACATAACG and GTCAGGAGCTCGAAAATTTATAAGATAATTCGTCCAAATGGC. The product was

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: SI, self-incompatible; S-RNases, S-locus RNases; SC, self-compatible.

Data deposition: The sequence reported in this manuscript has been deposited in the GenBank database (accession no. AF128405).

*To whom reprint requests should be addressed: E-mail mcclureb@missouri.edu.

†Present address: Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

‡Present address: Department of Pathology, University of Zürich, Schmelzbergstrasse 12, CH-8091 Zürich Switzerland.

§Present address: Department of Plant and Soil Science, University of Massachusetts, Amherst, MA 01003.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

digested and ligated to the cauliflower mosaic virus 35S promoter and *nos* 3′ sequences from pAGUS1 (15, 18). The HT-antisense construct was recloned in pZP122 (19), transferred to *Agrobacterium tumefaciens* GV3101, and used to transform *N. plumbaginifolia*. (*N. plumbaginifolia* × SC *N. alata*) hybrids previously transformed with pSC109617 (14), and expressing S_{C10}-RNases, were also transformed with the HT-antisense construct. Transformants were regenerated on gentamycin or gentamycin plus kanamycin. Twelve independent, doubly transformed (*N. plumbaginifolia* × SC *N. alata*) hybrids were analyzed as primary transformants. Then, 26 independently transformed *N. plumbaginifolia* lines were crossed to untransformed *N. alata* $S_{C10}S_{C10}$ and second-generation transgenic (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrids were analyzed for HT-expression and pollination phenotype (15).

RNA and Protein Blot Analysis. Organs were collected, frozen in liquid nitrogen, and stored at −70°C until needed. Total RNAs were prepared and separated in 2% agarose formaldehyde gels as described (5). RNAs were blotted onto Hybond N+ (Amersham) and stained with methylene blue to check for equal loading. Blots were hybridized to ^{32}P-labeled HT- or S_{C10}-RNase cDNAs. Stringent washes were performed in 0.15 × SSPE [standard saline phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)], 1% SDS, 1% powdered milk, and 4 mg/ml salmon testis DNA (Sigma, D-3159) at 68°C. Autoradiographs were prepared with Kodak XRP-5 films (Rochester, NY) at −70°C. In the experiment shown in Fig. 4, the results were quantitated with a Molecular Dynamics model 400A PhosphorImager and were normalized to the highest signal.

To generate the HT-antiserum, the HT-cDNA was engineered to create *Eco*RI and *Bam*HI sites flanking the nonreptitive part of the mature-coding sequence by using the synthetic oligonucleotides GAAGGATCCAGGGATATGGTTGATCCTTCAATATCATT and GAGGAATTCTTAACCCTTTTGGCATTTGCAAGCTGCAC. The ORF was cloned into pGEX2TK (Amersham), and a glutathione S-transferase-HT fusion was purified from *Escherichia coli* with glutathione agarose (Sigma, G-4510). After further purification by SDS/PAGE, the fusion protein was injected into a rabbit to raise an anti-HT serum (20). For protein blot analysis, styles were weighed, homogenized in SDS-loading buffer (0.2 M Tris·HCl, pH 6.8/0.5 M DTT/4% SDS/25% glycerol; 10 μl per mg of freshweight), boiled, and centrifuged. Proteins were separated in 10% Tris-Tricine gels (21) and blotted onto Nitrobind (Micron Separations, Westborough, MA) by using a Bio-Rad Transblot SD semi-dry electroblotting apparatus. Blots were treated with the rabbit HT-antiserum or a mouse monoclonal anti-S_{C10}-RNase Ab (14), and immune complexes were detected by using alkalinephosphatase-conjugated secondary antibodies and nitroblue tetrazolum/BCIP (5-bromo-4-chloro-3-indolyl phosphate) (20).

Pollination Phenotypes. Emasculated flowers were pollinated 1 day after petal opening with pollen from SI *N. alata* $S_{C10}S_{C10}$ or $S_{105}S_{105}$ as described (14). Styles were harvested after 72 hr, stained with decolorized aniline blue, and examined by epifluorescence (22). Whenever possible, the total number of pollen tubes penetrating to the base of the style was counted. However, when $>\approx 50$ pollen tubes were present, it was not possible to obtain accurate counts. Styles with >150 pollen tubes were scored as highly compatible (+++).

Results

The SC *N. alata* cultivar Breakthrough does not express an S-RNase, but our previous results show that it does express non-S-RNase factors required for pollen rejection (14). To clone these factors, a cDNA library was prepared by using mature style mRNA. Several sequences were identified in a differential screen as being highly expressed in SC *N. alata* but showing little or no signal with a *N. plumbaginifolia* probe. As a secondary screen, putative non-S-RNase factor clones were tested for hybridization to RNA from SI *N. alata*, SC *N. alata*, *N. plumbaginifolia*, and also *N. longiflora*, a second SC species that lacked factors required for S-allele-specific pollen rejection (B.M, unpublished data). Putative non-S-RNase factors are expected to be expressed in both accessions of *N. alata* but show little or no expression in *N. plumbaginifolia* or *N. longiflora*. Fig. 1 shows RNA blot results for a clone designated HT. The 600-nt HT-transcript is present in style RNA from SI *N. alata* $S_{105}S_{105}$ and SC *N. alata* but cannot be detected in either *N. plumbaginifolia* or *N. longiflora*, even after extended exposures.

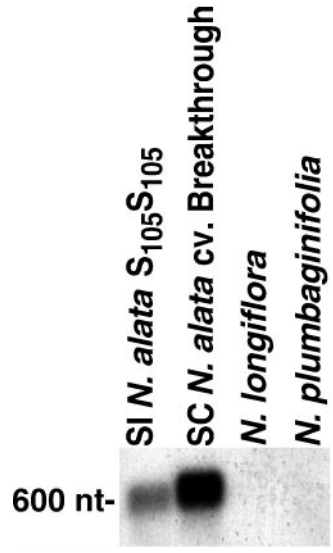

Fig. 1. HT-transcript accumulation in different genetic backgrounds. The 600-nt HT-transcript is only visible in SI *N. alata* $S_{105}S_{105}$ and SC *N. alata*, which both express a full complement of the non-S-RNase factors required for S-allele-specific pollen rejection. *N. plumbaginifolia* and *N. longiflora* represent backgrounds deficient in one or more such factors. Polyadenylated RNA from mature styles (200 ng/lane) was blotted and hybridized with ^{32}P-labeled HT-cDNA.

Fig. 2 shows the predicted 101-residue amino acid sequence of the HT-protein. A putative signal sequence is present at the N terminus, including a basic residue at position 4 followed by a series of hydrophobic residues. The SignalP algorithm (23) predicts the highest probability of signal sequence cleavage before Arg-24, leaving a mature polypeptide of 8.6 kDa. The mature HT-protein is predicted to be fairly acidic (i.e., calculated pI = 3.76 for residues 24–101, Fig. 2). In part, this is due to a striking stretch of 20 asparagine and aspartic acid residues near the C terminus. The asparagine-rich domain is flanked on each side by three cysteine residues that could be involved in disulfide bonding or posttranslation modification, but it is not yet known whether such modifications exist. The sequence does not contain potential *N*-glycosylation sites. Aside from homologies to the repetitive asparagine-rich domain, there were no significant

HT-protein sequence

```
malkanvlil slvlliisse viaRDMVDPS   30
ISLLEPNNDK KTNGMNDATL QKIGGKVGMF   60
FDFMCAACKC QKGNNDNNDN DNDNDNDNNN   90
NNDIVCQTVC C                      101
```

Fig. 2. Predicted amino acid sequence of HT-protein. The predicted secretion signal sequence is lowercase.

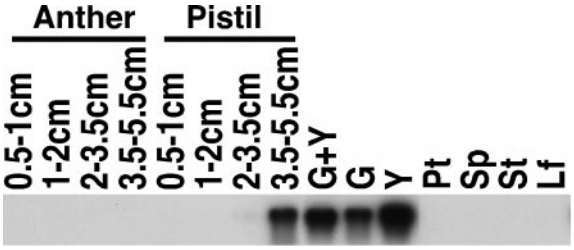

Fig. 3. HT-transcript accumulation in SC *N. alata* cv Breakthrough. Developing anthers (lanes 1–4) and styles (i.e., stigma plus style, lanes 5–8) were dissected from buds of the indicated sizes. G + Y, styles at anthesis; G, isolated stigmas at anthesis; Y, isolated styles at anthesis; Pt, petals; Sp, sepals; St, stems; Lf, leaf. Total RNAs (10 μg/lane) were blotted and hybridized with ^{32}P-labeled HT-cDNA.

matches to the HT-protein sequence in the databases (i.e., BLAST (24) score >30; search conducted in Feb. 1999).

Fig. 3 shows total RNA blot analysis of HT-transcript expression in various organs of SC *N. alata.* Anthers and pistils were removed from buds ranging from 0.5 to 5.5 cm. In SC *N. alata* cv. Breakthrough, the 0.5- to 1-cm stage includes anthers in tetrads and anthesis occurs in buds ≈5.5 cm in length. HT-transcript is not detectable at any stage of anther development. In the pistil (i.e., including the stigma and the style, but not the ovary), a low level of HT-transcript is first detected in 2- to 3.5-cm buds, and the amount of expression increases dramatically at maturity. Fig. 3 shows the highest level of HT-transcript present in anthesis-stage pistils. Stigmas and styles were dissected from anthesis-stage pistils, and Fig. 3 shows that HT-transcript is expressed in both organs, with a slightly higher level in the style. Fig. 3 shows no HT-transcript in four nonsexual organs: petals, sepals, stems, and leaves.

A similar experiment was performed to examine HT-transcript expression in SI *N. alata* $S_{C10}S_{C10}$. RNA blots were prepared and hybridized to S_{C10}-RNase or HT-cDNA probes. Fig. 4 shows that HT-transcript accumulation lags behind S_{C10}-RNase transcript accumulation. Whereas S_{C10}-RNase transcript is first detectable in 1- to 2-cm buds, HT-transcript is first detectable at the next stage, in 2.0- to 3.5-cm buds (Fig. 4 *Upper*). The hybridization results were quantified by using a PhosphorImager and normalized to the signal from mature style RNA (i.e., 5.5-cm anthesis, Fig. 4). The most critical stage is when the buds are 2.0–3.5 cm long because this is when the style becomes competent to support pollen tube growth but rejects S_{C10}-pollen poorly. At this stage, Fig. 4 shows that S_{C10}-RNase transcript levels are already 67% of their highest level, whereas HT-transcript levels are only 4% of the level in mature styles. By the next stage, when the pistil is fully competent to reject S_{C10}-pollen, HT-transcript levels show an eightfold increase (i.e., to 32% of the mature level, Fig. 4).

An antisense construct was prepared to test whether HT-protein is required for S-allele-specific pollen rejection. Initially, the construct was transformed into (*N. plumbaginifolia* × SC *N. alata*) hybrids expressing S_{C10}-RNase from pSC109617 (14). The results with these doubly transformed hybrids were promising. Controls showed normal S-allele-specific rejection of S_{C10}-pollen, but some antisense transformed plants showed changes in pollination phenotype (data not shown). To confirm these results with second generation plants, the HT-antisense construct was transformed into *N. plumbaginifolia* and then crossed with SI *N. alata* $S_{C10}S_{C10}$. Because *N. plumbaginifolia* is easier to transform than *N. alata*, this approach affords an opportunity to analyze a greater number of independently transformed lines than would be available from direct transformation of *N. alata*. We have previously used this approach to show that S-RNase

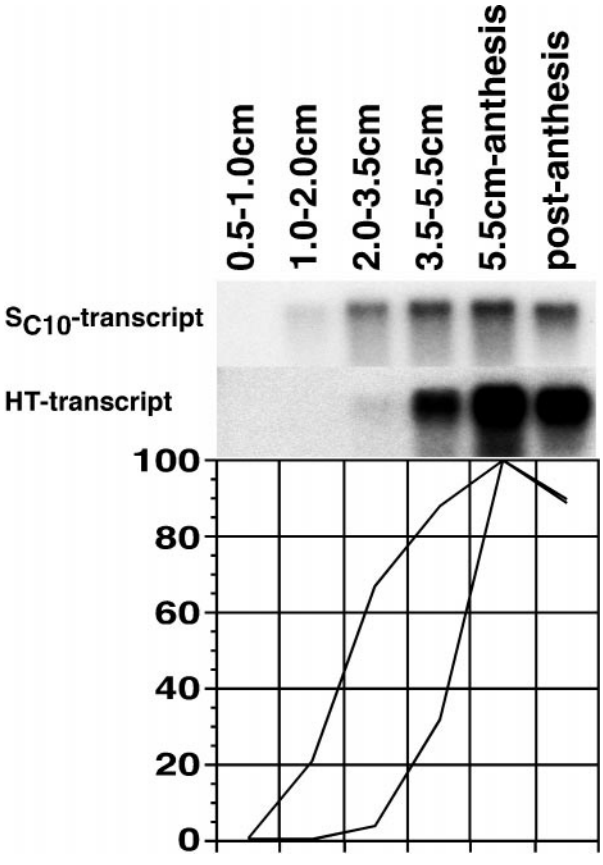

Fig. 4. HT-transcript accumulation in SI *N. alata* $S_{C10}S_{C10}$. Developing styles (i.e., stigma plus style) were dissected from buds of the indicated sizes. (*Upper*) Autoradiographs. (*Lower*) Signals quantified with a PhosphorImager and normalized to the highest signal. Total RNAs (5 μg per lane) were blotted and probed with ^{32}P-labeled HT- or S_{C10}-RNase probes.

antisense constructs suppress S-RNase expression and prevent S-allele-specific pollen rejection in the resulting hybrid [i.e., the second generation after transformation, (15)].

To facilitate analysis of the transformed plants, an HT-antiserum was prepared to a glutathione S-transferase-HT-protein fusion. The antiserum reacts with several stylar polypeptides that migrate with apparent M_r from 10 to 18 kDa. To confirm that these polypeptides correspond to HT-protein, they were partially purified from SI *N. alata* $S_{A2}S_{A2}$ and $S_{C10}S_{C10}$ material, blotted onto poly(vinylidene difluoride) and subjected to N-terminal sequencing. A band with apparent M_r of ≈18 kDa gave the sequence RDMVDPSISL, corresponding to the putative N terminus predicted by the SignalP algorithm (Fig. 2). Two slightly faster migrating bands isolated from SI *N. alata* $S_{C10}S_{C10}$ material, both gave the sequence KIGGKVGMFF, corresponding to residues 52–61 in Fig. 2. Thus, HT-protein may be subject to processing or degradation, but it is clear that the HT-antiserum is specific to HT-protein.

The HT-antiserum was used to examine HT-protein levels in hybrids transformed with the antisense construct. The results show that suppression of HT-protein expression interferes with S-allele-specific pollen rejection. Fig. 5 shows protein blot analysis of style extracts from SI *N. alata* $S_{C10}S_{C10}$, *N. plumbaginifolia*, an untransformed (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrid, and hybrid progeny from six independent antisense HT-transformed *N. plumbaginifolia* lines. The positive controls, SI *N. alata* $S_{C10}S_{C10}$ and the untransformed hybrid, both express HT-protein and S_{C10}-RNase. *N. plumbaginifolia*, the negative

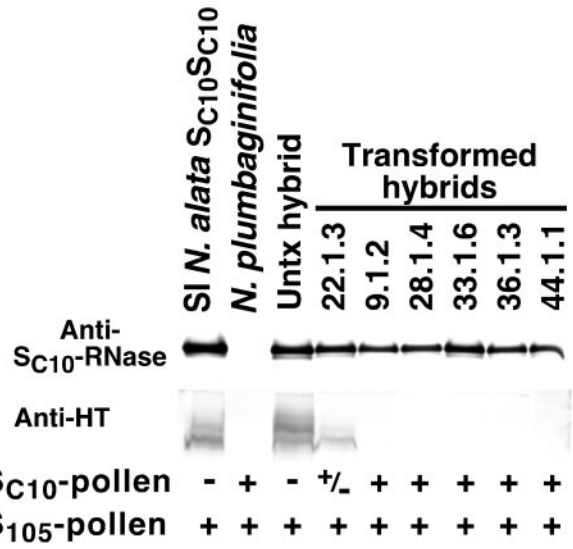

Fig. 5. Protein blot analysis of HT-antisense transformed (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrids. SI *N. alata* $S_{C10}S_{C10}$ and the untransformed (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrid are positive controls showing expression of S_{C10}-RNase, HT-protein, and a normal S-allele-specific pollen rejection phenotype. Untransformed *N. plumbaginifolia* is a negative control expressing neither protein and accepts both types of pollen. The transformed hybrids represent progeny from six independently transformed HT-antisense *N. plumbaginifolia* lines. Style extracts equivalent to 0.33 mg (anti S_{C10}-RNase) or 2.5 mg of freshweight (anti-HT) were blotted and immunostained. Pollination phenotypes were tested using pollen from *N. alata* S_{C10}- or S_{105}-pollen (Table 1) and are summarized here as +, compatible; −, incompatible; or +/−, semi-compatible.

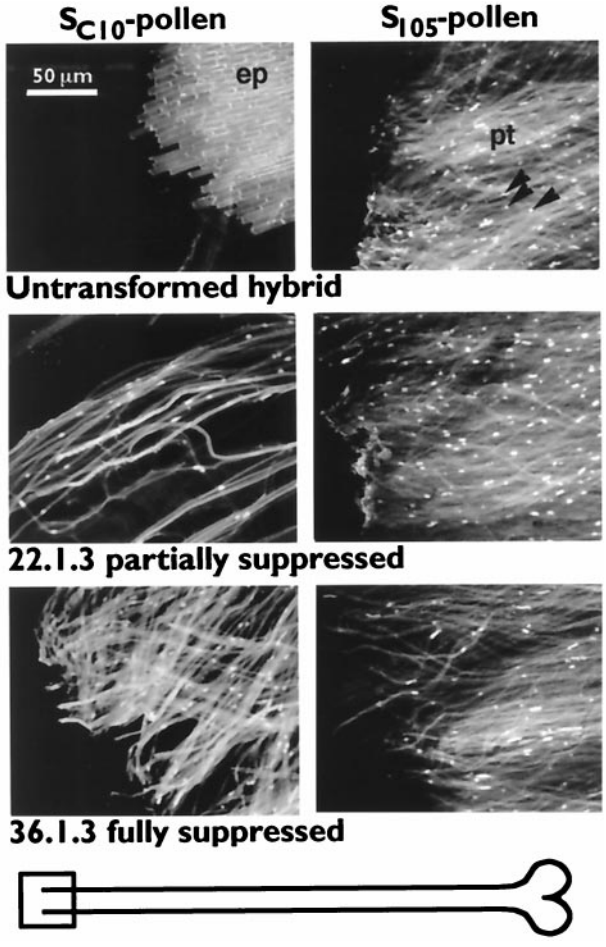

Fig. 6. Pollen tube staining results for HT-antisense transformed (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrids. Hybrids were pollinated with S_{C10}- or S_{105}-pollen and prepared for epifluorescence microscopy after 72 hr. As shown in the diagram, the field of view is at or very near the base of the style. No pollen tubes are visible at the base of the style after pollinating untransformed hybrids with S_{C10}-pollen but a fragment of epidermis (ep) is shown for orientation. Pollen tubes (pt) appear as fibers with brightly stained callose plugs (arrows). The untransformed control hybrid shows normal S-allele-specific pollen rejection because no S_{C10}-pollen tubes are evident but many S_{105}-pollen tubes can be seen at the base of the style. Approximately 20 S_{C10}-pollen tubes are visible in the partially suppressed hybrid (22.1.3). Numerous S_{C10}-pollen tubes are visible in fully suppressed hybrids such as 33.1.3, indicating loss of S-allele-specific pollen rejection.

control, does not express either protein and does not show S-allele-specific pollen rejection. The transformed hybrids all express S_{C10}-RNase, but five (i.e., 9.1.2, 28.1.4, 33.1.6, 36.1.3, and 44.1.1) show no detectable HT-protein expression, and hybrid 22.1.3 shows partial suppression.

Table 1 contains the results from at least five pollinations on each transgenic hybrid (results also summarized in Fig. 5). Fig. 6 shows examples of the style squashes used to score the pollination phenotypes. Because pollen tubes become crowded and tangled, styles with >150 pollen tubes at the bottom of the style were scored as "uncountable" or highly compatible (cf. S_{105}-pollen results in Fig. 6; +++, Table 1). Untransformed control (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrids showed a normal S-allele-specific pollen rejection response. Thirteen pollinations with S_{105}-pollen were scored as +++ (i.e., uncountable), but only two pollen tubes were observed in 10 styles pollinated with S_{C10}-pollen. Broadly, the number of S_{C10}-pollen tubes that penetrate to the base of the style is inversely correlated with the level of HT-protein expression seen in Fig. 5. For example, the partially suppressed hybrid consistently showed several (i.e., 11–27, Table 1 and Fig. 6) S_{C10}-pollen tubes at the base of the style 72-h after pollination. Two fully suppressed hybrids (i.e., 28.1.4 and 33.1.6, Fig. 5) were scored as uncountable in every pollination. Three others (i.e., 9.1.2, 36.1.3, and 44.1.1; Fig. 5) showed a reduced number of S_{C10}-pollen tubes

Table 1. Pollen tubes at the base of the style 72 h postpollination in antisense-HT-transformed (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrids

Pistil	S_{105}-pollen positive control	S_{C10}-pollen test
22.1.3	+++, +++, +++, +++, ~150	27, 19, 11, 15, 19, 27
9.1.2	+++, +++, +++, +++, +++	+++, +++, +++, 34, 26
28.1.4	+++, +++, +++, +++, +++, +++	+++, +++, +++, +++, +++
33.1.6	+++, +++, +++, +++, +++, +++	+++, +++, +++, +++, +++, +++
36.1.3	+++, +++, +++, +++, +++	+++, +++, +++, 45, 32
44.1.1	+++, +++, +++, +++, +++	+++, 100, 80, 29, 22

in at least some pollinations. Thus, the ability to specifically reject S_{C10}-pollen is suppressed in these transformed hybrids. When HT-protein levels are reduced, pollination with S_{C10}-pollen comes to resemble pollination with S_{105}-pollen (Fig. 6).

Discussion

Because *N. plumbaginifolia* and *N. alata* are closely related, and have similarities in their pollination behavior, we reasoned that most of the genes expressed in their styles would be highly homologous. We therefore used a differential screen to identify candidate non-S-RNase factors expressed in SC *N. alata*, but not in *N. plumbaginifolia*, that might be required for S-allele-specific pollen rejection. Among the selected cDNAs, only the HT clone showed a qualitative difference in expression between *N. plumbaginifolia* and SC *N. alata*. Further experiments showed that the 600-nt HT-transcript is expressed in SI *N. alata* $S_{105}S_{105}$ but not in a SC *N. longiflora* accession (Fig. 1). Expression in the SI background is consistent with a role in S-allele-specific pollen rejection. *N. longiflora* was tested because, like *N. plumbaginifolia*, it appears to lack one or more factors required for S-RNase-dependent pollen rejection (B. Mou and B. McClure, unpublished data). The accession used here expresses an S-RNase-like protein, but it is SC and also accepts pollen from *N. plumbaginifolia*. However, (*N. longiflora* × SC *N. alata*) hybrids reject pollen from *N. longiflora* and from *N. plumbaginifolia*, suggesting that the S-RNase-like protein can function when expressed in conjunction with factors from SC *N. alata*. This result is identical to the behavior of transgenic *N. plumbaginifolia* and (*N. plumbaginifolia* × SC *N. alata*) hybrids expressing S-RNase (14). Thus, the results in Fig. 1 show that HT-transcripts are present in two *N. alata* accessions known to possess a full complement of factors required for S-allele-specific pollen rejection and are not expressed in two SC species known to be defective for one or more such factors.

The 101-residue polypeptide predicted from the HT-cDNA sequence contains a stretch of 20 asparagine and aspartate residues near the C terminus. Asparagine-rich domains occur in genes from diverse organisms, but no general function has been ascribed to them. Some ''nonclassical'' arabinogalactan protein genes contain asparagine-rich domains (25, 26). Interestingly, the asparagine-rich domains inferred from arabinogalactan protein-cDNA sequences were not present in the isolated proteins, suggesting that they were removed *in vivo*. Unfortunately, aside from homologies to the repetitive asparagine-rich domain, there are no clearly homologous sequences in the databases to suggest a function for the HT-protein. However, it is noteworthy that HT-protein is predicted to be acidic, so it could interact with basic proteins such as S-RNase, although no such direct interaction has yet been detected.

The HT-protein was identified in style extracts by using an HT-antiserum raised against a glutathione *S*-transferase-HT fusion expressed in *E. coli*. The most prominent immunoreactive species in style extracts migrate with an apparent M_r of ≈18 kDa. This is considerably larger than the size predicted from the cDNA sequence, but N-terminal sequencing confirmed that these polypeptides are derived from the HT sequence. The aberrant migration may be due to posttranslational modification. Sequence from the 18-kDa band matched the N-terminal sequence predicted by the SignalP program suggesting that the HT-protein is secreted (i.e., RDMVDPSISL, Fig. 2). However, the HT-protein is unstable in style extracts (B.M., unpublished data), and faster migrating bands showed sequences derived from internal HT-protein regions (i.e., KIGGKVGMFF, Fig. 2). Thus, the conclusion that the HT-protein is secreted should be regarded as tentative until it is confirmed by another method.

The pattern of HT-transcript expression is consistent with a role in S-allele-specific pollen rejection. In SC *N. alata*, the transcript is not detectable in anthers or in nonsexual organs, but it is expressed in mature stigmas and the styles (Fig. 3). In SI *N. alata* $S_{C10}S_{C10}$, HT-transcript accumulation lags slightly behind S_{C10}-RNase transcript accumulation (Fig. 4). The flowers in this SI accession are slightly larger than in cv. Breakthrough, tetrads are visible in anthers from 0.8-cm buds, and the flowers open when they are 6- to 7-cm long. Bud-selfing is only successful in buds 2.5-cm long, suggesting that the pistil has just become competent to support pollen tube growth. Slightly longer buds reject S_{C10}-pollen. At this critical time (i.e., 2.0- to 3.5-cm buds, Fig. 4), S_{C10}-RNase expression has reached two-thirds of its maximum level, but HT-transcript levels are very low. The subsequent rapid increase in HT-transcript is strongly correlated with the onset of S-allele-specific pollen rejection (Fig. 4).

An antisense construct was prepared from the HT-cDNA, and its effect on pollination phenotype was tested in (*N. plumbaginifolia* × SI *N. alata* $S_{C10}S_{C10}$) hybrids. These hybrids are sterile so pollination phenotypes were assessed by examining style squashes stained with decolorized aniline blue. HT-protein and S_{C10}-RNase levels were monitored in control and transgenic hybrids by immunostaining style extracts. Untransformed hybrids express both proteins and show S-allele-specific pollen rejection; few S_{C10}-pollen tubes penetrate to the base of the style, but the hybrids are compatible with S_{105}-pollen (Fig. 5, Table 1). Suppressed HT-expression is correlated with loss of the ability to reject S_{C10}-pollen in the transgenic hybrids (Fig. 5, Table 1). S_{C10}-RNase levels in the transgenic hybrids were similar to the levels in the untransformed control. Therefore, we conclude that loss of S-allele-specific pollen rejection in the transgenic hybrids is due to reduced expression of HT-protein.

Although these results implicate HT-protein in S-allele-specific pollen rejection, its precise function is unknown. Clearly, it is incapable of causing pollen rejection on its own because it is expressed in SC *N. alata*. The variability in S_{C10}-RNase expression in transgenic hybrids with suppressed HT-expression (Fig. 5) is intriguing. However, HT-protein cannot be required for S-RNase expression *per se* because it is possible to achieve high level S-RNase expression in *N. plumbaginifolia*, which does not express detectable HT-protein. Moreover, HT-transcript accumulation begins after S_{C10}-RNase transcript (Fig. 4). We have not been able to purify enough HT-protein to quantify expression at the protein level. However, even allowing for the fact that it appears to be unstable in style extracts (B. McClure, unpublished data), it is unlikely that HT-protein is stoichiometric with S-RNase, which is expressed at near millimolar levels (27). These observations suggest that it is unlikely that HT-protein and S-RNase form a complex that is active in pollen rejection. It remains possible that HT-protein and S-RNase interact in some other way. It is also possible that HT-protein interacts only with pollen tubes, perhaps facilitating S-RNase uptake.

Although its exact function is unknown, our data show that HT-protein is implicated in S-allele-specific pollen rejection. We are currently working toward identification of further non-S-RNase factors required in style-part SI functions. It is likely that several such factors exist. However, we still know nothing about the pollen-part specificity determinant (pollen-S), or whether additional factors, beyond the specificity determinant, may be required for pollen SI functions. As more factors in the SI pathway are characterized, it should be possible to define the functions of individual factors more precisely.

We thank Melody Kroll and Waheeda Sulaman for technical assistance. This work was supported by the Swiss National Science Foundation, the University of Missouri Research Board, the Food for the 21st Century Program, and U.S. National Science Foundation Grants 93–16152 and 96–04645.

1. de Nettancourt, D. (1977) *Incompatibility in Angiosperms*. *Monographs on Theoretical and Applied Genetics 3* (Springer, Berlin).
2. Franklin, F. C. H., Lawrence, M. J. & Franklin-Tong, V. E. (1995) *Int. Rev. Cytol.* **158,** 1–64.
3. McClure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Simpson, R. J., Sakiyama, F. & Clarke, A. (1989) *Nature (London)* **342,** 955–957.
4. Huang, S., Lee, H.-S., Karunanandaa, B. & Kao, T.-h. (1994) *Plant Cell* **6,** 1021–1028.
5. McClure, B. A., Gray, J. E., Anderson, M. A. & Clarke, A. E. (1990) *Nature (London)* **347,** 757–760.
6. Gray, J. E., McClure, B. A., Bonig, I., Anderson, M. A. & Clarke, A. E. (1991) *Plant Cell* **3,** 271–283.
7. Ikeda, S., Nasrallah, J. B., Dixit, R., Preiss, S. & Nasrallah, M. E. (1997) *Science* **276,** 1564–1566.
8. Bower, M. S., Matias, D. D., Fernandes-Carvalho, E., Mazzurco, M., Gu, T., Rothstein, S. J. & Goring, D. R. (1996) *Plant Cell* **8,** 1641–1650.
9. Hearn, M., Franklin, F. C. H. & Ride, J. P. (1996) *Plant J.* **9,** 467–475.
10. Gu, T., Mazzurco, M., Sulaman, W., Matias, D. D. & Goring, D. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 382–387.
11. East, E. M. (1932) *Genetics* **17,** 175–202.
12. Bernatzky, R., Glaven, R. H. & Rivers, B. A. (1995) *Biochem. Genet.* **33,** 215–225.
13. Ai, Y., Kron, E. & Kao, T.-h. (1991) *Mol. Gen. Genet.* **230,** 353–358.
14. Murfett, J. M., Strabala, T. J., Zurek, D. M., Mou, B., Beecher, B. & McClure, B. A. (1996) *Plant Cell* **8,** 943–958.
15. Murfett, J., Bourque, J. E. & McClure, B. A. (1995) *Plant Mol. Biol.* **29,** 210–212.
16. Zurek, D. M., Mou, B., Beecher, B. & McClure, B. (1996) *Plant J.* **11,** 797–808.
17. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G. & Fraley, R. T. (1985) *Science* **227,** 1229–1231.
18. Skuzeski, J. M., Nichols, L. M. & Gesteland, R. F. (1990) *Plant Mol. Biol.* **15,** 65–69.
19. Hajdukiewicz, P., Svab, Z. & Maliga, P. (1994) *Plant Mol. Biol.* **25,** 989–994.
20. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
21. Schägger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379.
22. Kho, Y. O. & Baer, J. (1968) *Euphytica* **17,** 299–302.
23. Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997) *Protein Eng.* **10,** 1–6.
24. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Miller, W. & Lipman, D. J. (1997) *Nucleic Acids Res.* **17,** 3389–3402.
25. Mau, S.-L., Chen, C.-G., Pu, Z.-Y., Moritz, R. L., Simpson, R. J., Bacic, A. & Clarke, A. E. (1995) *Plant J.* **8,** 269–281.
26. Du, H., Clarke, A. E. & Bacic, A. (1996) *Trends Cell Biol.* **6,** 411–414.
27. Jahnen, W., Batterham, M. P., Clarke, A. E., Moritz, R. L. & Simpson, R. J. (1989) *Plant Cell* **1,** 493–499.