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S-RNase Gene of *Nicotiana alata* Is Expressed in Developing Pollen

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In the solanaceous plant *Nicotiana alata*, self-incompatibility is controlled by a single, multiallelic locus (*S* locus) expressed in both pollen and pistil. Previously, we have shown cosegregation between alleles of the *S* locus and alleles of a gene that encodes a glycoprotein with ribonuclease activity (*S*-RNase). Furthermore, expression of the *S*-RNase gene is apparently confined to the pistil and is correlated with the onset of self-incompatibility. In this paper, we report that the *S*-RNase gene is also expressed at low levels in developing pollen. A transcript in developing pollen hybridized to a cDNA encoding the S_2-RNase allele of the parent plant and did not hybridize to cDNAs encoding other *S*-RNase alleles. Two cDNAs for the S_2-RNase were cloned from a library derived from anthers of a plant homozygous for the S_2 allele and both corresponded to the coding sequence of the S_2-RNase. The product of the *S*-RNase gene was detected by immunocytochemistry in the intine of mature, hydrated pollen grains. These results are interpreted in the light of current knowledge of the structure of the *S* locus.

INTRODUCTION

Self-incompatibility (SI) is a genetically controlled mechanism that prevents inbreeding in many families of flowering plants (de Nettancourt, 1977). In many dioecious plants, SI is determined by a single locus (the *S* locus) with multiple alleles. At least two distinct systems exist: the sporophytic system, found in the Brassicaceae (Dzelzkalns et al., 1992), in which the SI phenotype of the pollen is determined by the diploid genotype of its parent and the gametophytic system, found in the Solanaceae (Haring et al., 1990), in which the pollen phenotype is determined by its own haploid genotype.

The gametophytic SI system involves expression of the *S* locus in pollen and pistil and the recognition and rejection of self-pollen by the pistil. Lewis (1949, 1960), using mutations induced by radiation, showed that the *S* locus could be affected independently in the pollen and style of self-incompatible *Oenothera organensis.* This finding was confirmed later in similar experiments by Pandey (1965), working with *Nicotiana alata.* The two parts of the *S* locus could represent a single gene with separate promoter elements directing expression in pollen or style or two closely linked genes in which one gene is expressed in each tissue.

A number of alleles of a gene that segregates with the *S* locus have been cloned from solanaceous species (Anderson et al., 1986; Ai et al., 1990; Clark et al., 1990; Xu et al., 1990; Kaufmann et al., 1991). These genes encode basic glycoproteins of ~30 kD with ribonuclease activity, which have been termed *S*-RNases. The *S*-RNase genes are highly expressed in the pistils of self-incompatible flowers, coincident with the onset of SI. *S*-RNases are found in the transmitting tract of the style along the path through which pollen tubes must grow to reach the ovaries. The *S*-RNases are believed to be the determinant of the stylar SI phenotype of solanaceous self-incompatible plants.

The nature of the pollen component of the *S* locus and its relationship to the style *S*-RNase are the focus of current efforts to understand the mechanism of gametophytic SI. So far, attempts to detect expression of *S*-RNases in pollen or pollen tubes have been unsuccessful (Clark et al., 1990; Haring et al., 1990; Mau et al., 1991). The lack of evidence for pollen expression of the *S*-RNase favors the hypothesis that the pollen component of the *S* locus is encoded by a separate but closely linked gene.

We present evidence that the *N. alata S*-RNase gene is expressed during pollen development and discuss this finding in the context of theories regarding the structure of the *S* locus.

RESULTS

Stages of Pollen Development in Anthers from Buds of Various Lengths

Anthers of *N. alata* were examined at three stages of development that were defined by the length of the flower bud. Figure

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1 shows light micrographs of anther sections at each of these stages. Because pollen development throughout the anther is not synchronous, each stage represents a range of developmental states of pollen. Figure 1A shows a longitudinal section of an anther from a 10-mm bud (stage 1) stained with acridine orange. The tapetal layer is stained bright orange, reflecting the high content of RNA. The outer epidermal layers of the anther are weakly stained. The microspores located centrally within the pollen sac exhibit various staining patterns. A higher magnification of pollen in an anther at this stage (Figure 1B) shows pollen at several early stages of development. Condensed chromosomes (stained bright yellow) are visible in some cells that are engaged in meiosis, whereas most cells have completed meiosis and are characterized by orange-staining cytoplasm containing RNA. Other cells at a more advanced stage of development have large vacuoles and show some degradation of premeiotic RNA.

Table 1. State of Pollen in Anthers of *N. alata* at Three Stages of Development

Stage	Bud Length (mm)	State of Pollen Development
1	5–10	Late meiosis, tetrad formation, early vacuole formation and RNA degradation. Active tapetum.
2	20–30	Vacuolate stage, little RNA, uninucleate and binucleate pollen. Tapetum less active.
3	50–70	Pollen near maturity. Tapetum degraded.

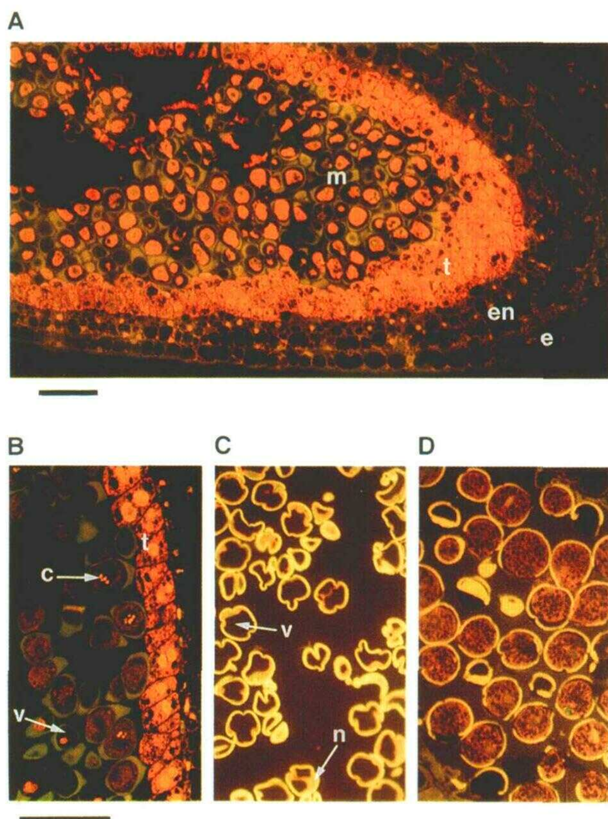

Figure 1. Light Micrographs of Sections of Developing Anthers of *N. alata* Stained with Acridine Orange and Visualized by UV Fluorescence.

(A) Section of an anther from a 10-mm bud showing the tapetal layer (t), microspores (m), epidermis (e), and endothecium (en). RNA is stained orange; cell walls, green; and condensed chromosomes, bright yellow.

(B) Microspores in an anther from a 10-mm bud. A line of tapetal cells (t) is visible adjacent to the pollen microspores; some of the cells contain condensed chromosomes (c) or vacuoles (v).

(C) Microspores in an anther from a 25-mm bud. Dark vacuoles (v) and nuclei (n) are visible.

(D) Microspores in an anther from a 50-mm bud.

Bars = 50 μm.

Figures 1C and 1D show sections of stage 2 and 3 anthers (bud length 25 and 50 mm, respectively). Stage 2 anthers contain mainly vacuolate pollen with little RNA detected by staining; the premeiotic RNA has been degraded and the gametophytic RNA is beginning to be synthesized. Microspore mitosis has occurred in some cells, and both uninucleate and binucleate pollen cells are present (I. Bönig, unpublished results). Stage 3 anthers contain pollen that is close to maturity and is densely packed with RNA, which has been synthesized postmeiotically. The tapetal layer of the anther has been completely degraded by this stage. The three stages are summarized in Table 1.

Detection of RNA with Homology to the Style S_2-RNase in Developing Anthers by RNA Gel Blot Analysis

Total RNA was isolated from anthers at the three stages of development and from styles, stigmas, ovaries, mature pollen, stems, petals, and leaves of *N. alata* (genotype S_2S_2). Poly(A)$^+$ RNA was also isolated from in vitro–grown pollen tubes. Figure 2 shows the result of an RNA gel blot analysis performed on these samples using the S_2-RNase cDNA clone as a probe. Expression of the S_2-RNase mRNA was detected in the tissues of the pistil as a strongly hybridizing band (~950 nucleotides) after a 72-hr exposure to film. This overexposed the signals from the pistil-derived RNA samples, which were easily detectable after 3 hr (data not shown), but was required to detect a much weaker signal (much less than 1% of style signal) of a similar size in anthers at developmental stages 2 and 3. Comparison of the 3- and 72-hr exposures of this hybridization indicated that the message found in anther was slightly smaller (20 to 30 bp) than the style message. No cross-hybridization of this band to an S_6 cDNA probe was detected

(data not shown). No signal was detected in mature pollen, pollen tubes, or the vegetative tissues.

Detection of RNA with Homology to Style *S*-RNases in Developing Anthers by Polymerase Chain Reaction

We used a polymerase chain reaction (PCR)–based technique to detect expression of *S*-RNase homologs in developing anthers. Primers pC2 and pC3, based on the sequences of the conserved regions C2 and C3 of the *S*-RNase sequence, were synthesized. Figure 3 shows the location and orientation of these primers relative to the S_2-RNase protein sequence. The length of the predicted PCR product of these primers on an *S*-RNase cDNA template is 208 bp, and this product includes the site of an intron in the corresponding genomic sequence. PCR with genomic and cDNA clones of the S_2- and S_6-RNase genes produced fragments of the predicted lengths (data not shown), and the genomic products were easily distinguished from the cDNA products by their greater length.

First-strand cDNA synthesis primed with oligo(dT) was performed on total RNA samples from various tissues. These cDNAs were used as templates in PCRs with oligonucleotide primers pC2 and pC3. Figure 4A shows the PCR products obtained from these cDNAs, which were analyzed by agarose gel electrophoresis. In numerous experiments, products of the predicted length (208 bp) were always obtained from the female tissues style, stigma, and ovary, as well as from anthers at stages 2 and 3. A product was also obtained from pollen cDNA, but the amount of product was variable (usually low). Small amounts of product were also detected in stage 1 anthers, pollen tubes, and leaves. However, these results were not reproducible. When the RNA samples were used directly in the PCR reaction without cDNA synthesis, no products were detected (data not shown).

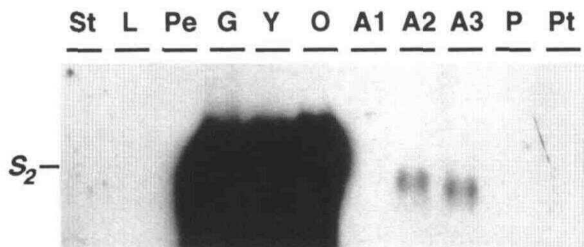

Figure 2. RNA Gel Blot Analysis of Transcripts Homologous to the S_2-RNase from Organs of *N. alata* (Genotype S_2S_2).

RNA samples from stem (St), leaf (L), petal (Pe), stigma (G), style (Y), ovary (O), anthers at stages 1, 2, and 3 (A1, A2, and A3), mature pollen (P), and pollen tubes (Pt) were separated on a 2% agarose gel containing 7% formaldehyde and hybridized to a ^{32}P-labeled S_2 cDNA probe. Lanes contain 10 µg of total RNA, except lanes G and Y, which contain 2.5 µg of total RNA, and lane Pt, which contains 200 ng of poly(A)$^+$ RNA. Autoradiograms were exposed at −70°C for 72 hr.

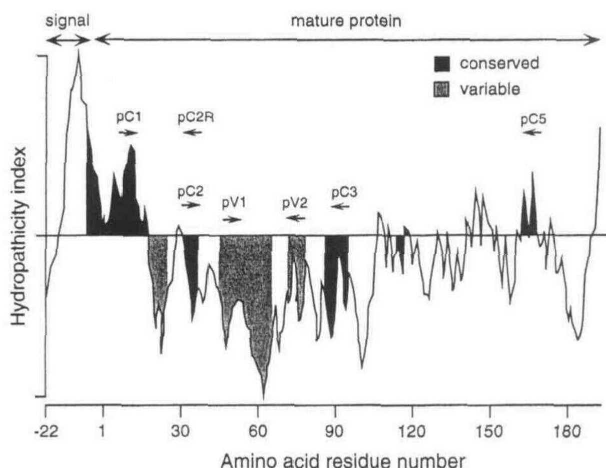

Figure 3. Positions of PCR Primers in the *S*-RNase Coding Region.

Hydropathy plot based on the *N. alata* S_2-RNase sequence and its alignment with cDNAs encoding other *S*-RNases from *N. alata*, petunia, and potato (Anderson et al., 1989; Clark et al., 1990; Kaufmann et al., 1991). The variable regions are shown in gray and the conserved regions in black. The position and orientation of each PCR primer are indicated by an arrow. The arrows labeled pC1, pC2, pC2R, pC3, and pC5 represent primers directed to conserved sequences within the *S*-RNase coding sequence. The arrows labeled pV1 and pV2 represent the primers pV1.2 and pV1.6, and pV2.2 and pV2.6, respectively. These primers are directed to sequences that are not conserved between S_2 and S_6 of *N. alata*; primers pV1.6 and pV2.6 match the S_6 sequence, while pV1.2 and pV2.2 match the S_2 sequence.

To test for *S* allele specificity, the PCR products from styles and stage 3 anthers of both genotypes were gel purified, and dilutions of these products were used as templates in a second PCR experiment. This experiment used primers directed to the variable regions of the *S*-RNase sequence, which are within the expected product of the first PCR reaction (Figure 3; pV1 and pV2). Two pairs of primers were used in separate reactions; one pair (pV1.6 and pV2.6) was directed to the variable region of the S_6 allele sequence, and the other pair (pV1.2 and pV2.2) was directed to the S_2 allele sequence (see Methods for primer sequences). In PCRs on the S_2- and S_6-RNase cDNA clones, products of the expected size were obtained in an allele-specific manner; that is, the S_6-specific primers generated a product from the S_6 template but not the S_2 template and vice versa for the S_2-specific primers (data not shown).

When the S_6-specific primers were used in PCRs with the purified PCR products primed by pC2 and pC3 (Figure 4B), a fragment of the expected 100-bp length was generated from the products of the first PCR on style and anther cDNAs from plants of genotype S_6S_6. No product was detected when the pC2 and pC3 PCR products from the style and anther of S_2S_2 plants were used. Conversely, when the S_2-specific primers were used (Figure 4B), a 100-bp product was produced from

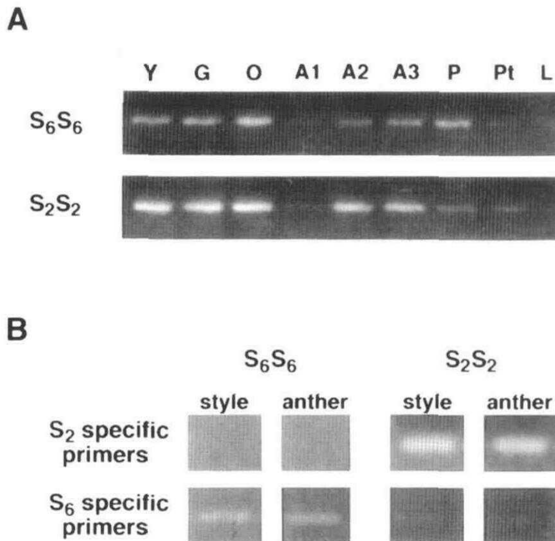

Figure 4. PCR Analysis of *S*-RNase Expression in Organs of *N. alata* (Genotypes S_2S_2 and S_6S_6).

(A) The primers pC2 and pC3 were used in a PCR reaction using cDNA from the style (Y), stigma (G), ovary (O), anthers at stages 1, 2, and 3 (A1, A2, and A3), pollen (P), pollen tubes (Pt), and leaves (L) of plants homozygous for the S_2 and S_6 alleles. The cDNA was produced using oligo(dT) primers, and each PCR used cDNA derived from 0.25 μg of RNA. The PCR products were run on a 3% NuSieve agarose gel and visualized by ethidium bromide staining. The length of the products is 210 bp.

(B) The pC2 and pC3 PCR products from S_2S_2 and S_6S_6 style and stage 3 anthers (anther) were gel purified and then used as templates for PCR reactions involving the primer pairs pV1.6 and pV2.6 (S_6 specific) and pV1.2 and pV2.2 (S_2 specific). PCR products were run on 3% Seaplaque LMP agarose and visualized by ethidium bromide staining. The length of the products is 100 bp.

the S_2S_2-derived first PCR products but not from the corresponding S_6S_6 products. To confirm this allele specificity, the pC2 and pC3 PCR products were cloned into pBluescript SK+ (modified as described in Methods). Several of these clones, each representing a single PCR fragment, were sequenced. Of eight clones obtained from the PCR products of S_2S_2 stage 3 anthers, all contained inserts corresponding to the S_2-RNase sequence. Two of the clones were identical in sequence to S_2-RNase, whereas eight single-base mismatches found in the remaining six clones may have resulted from errors introduced by Taq polymerase. Three of four clones derived from the S_6S_6 stage 3 anther PCR product were identical to the S_6-RNase sequence, whereas a fourth clone contained an insert with similarity to an RNase from tomato that is secreted by cultured tomato cells in response to phosphate starvation (Jost et al., 1991; results not shown).

In further PCR experiments, primers directed to other regions of the *S*-RNase sequence (Figure 3) were used on cDNAs prepared from the same RNA samples, using the 3′ primer of the pair to prime cDNA synthesis. The results of these reactions are summarized in Table 2. The predicted products of these primers span most of the mature polypeptide from amino acid residues 6 to 167, but do not include the N-terminal signal sequence. DNA fragments of the predicted sizes were produced from cDNAs derived from stylar RNA and from stage 2 and 3 anthers. In some, but not all experiments, a product is also observed from pollen cDNAs. Notably, S_6-specific primers resulted in a product when applied to S_6S_6-derived cDNAs but not those derived from S_2S_2 tissues, and vice versa.

One set of primers gave rise to products of anomalous sizes. The primer pair pC1 and pC3 gave rise to a product of 800 bp in PCR reactions on cDNAs from each of the anther stages and from pollen but gave the expected length of 271 bp on style cDNA. However, when the two primer pairs pC1 and pC2R

Table 2. Summary of Results of PCR Experiments Using Different Primer Combinations[a]

Primer Combination	Predicted Length (bp)	Template cDNA S_6S_6 Y	A1	A2	A3	P	L	S_2S_2 Y	A1	A2	A3	P	L
pC2/pC3	208	+	–	+	+	+	–	+	–	+	+	+	–
pV1.6/pV2.6	101	+	–	+	+	–	–	–	–	–	–	–	–
pV1.2/pV2.2	108	–	–	–	–	–	–	+	–	+	+	–	–
pV1.6/pC3	150	+	–	+	+	+	–	–	nd	nd	nd	nd	nd
pV1.2/pC3	157	–	nd	nd	nd	nd	nd	+	–	+	+	–	–
pC2/pC5	423	+	–	+	+	–	–	+	–	+	+	–	–
pC1/pC3	271	+	*	*	*	*	–	+	*	*	*	*	–
pC1/pC2R	88	+	nd	nd	+	nd	nd	+	nd	nd	+	nd	nd

[a] RNA from style (Y), stage 1, 2, and 3 anthers (A1, A2, and A3), pollen (P), and leaves (L) of both S_2S_2 and S_6S_6 genotypes was used to make cDNA primed by the downstream oligonucleotide of the PCR pair. The cDNA was used as the template in PCR reactions involving the stated primer pairs (Figure 3). Predicted lengths for each pair are indicated. Each reaction used cDNA derived from 2.5 μg of RNA. The results of the reactions are indicated as follows: +, product of correct length observed; –, no product observed; nd, not determined; asterisk, product of anomalous length detected (see text).

and pC2 and pC3 (which together span the same region as pC1 and pC3) were used, both resulted in products of the predicted size. The anomalous pC1 and pC3 product did not hybridize to a mixed S_2/S_6 cDNA probe on a DNA gel blot, whereas the style product hybridized strongly (data not shown).

Detection and Localization of RNA with Homology to the Style S-RNases in Developing Anthers by In Situ Hybridization

To localize the site of S-RNase expression within the anther, cryostat sections of stage 1, 2, and 3 anthers were prepared from flowers of S_2S_2 and S_6S_6 genotypes, fixed, and hybridized with a ^{32}P-labeled DNA probe. The probes used were gel-purified PCR fragments produced using the pC2 and pC3 primers with either the S_2 or S_6 cDNA clones as templates. These fragments span the variable region of the S-RNase sequence and show no cross-reaction when used to probe DNA dot blots of the S_2 and S_6 cDNA clones (data not shown). A strong, allele-specific signal was detected from style sections hybridized with the PCR probes (data not shown).

Figure 5A shows the hybridization of the S_2-specific probe to the anther sections. No signal was detected with this probe on anther sections taken from S_6S_6 flowers. However, for anthers of S_2S_2 genotype, weak signals were detected on sections of stage 1 and 2 anthers, and stage 3 anthers gave a strong signal. Complementary results were obtained when an S_6 probe was hybridized to similar anther sections (data not shown).

Figure 5B shows a light micrograph of a stage 3 anther section (genotype S_2S_2) stained with toluidine blue, alongside the hybridization pattern of the following section from the same anther. The hybridization of the S_2 probe was confined to the pollen sac and was not detected in the sporophytic tissue of the anther.

Isolation of cDNA Clones from a Library Constructed from Stage 3 Anther mRNA

Poly(A)$^+$ RNA was isolated from stage 3 anthers of S_2S_2 genotype and used to construct a cDNA library in λZAPII. The library was screened with a ^{32}P-labeled DNA probe produced by PCR from the S_2 cDNA clone using the primers pC1 and pC5. Two hybridizing plaques were detected from 1.9×10^5 independent clones. These two clones were excised as pBluescript SK+ plasmids and sequenced. Figure 6 shows an alignment of the sequences of these clones to the published sequence of an S_2 cDNA of *N. alata* (Anderson et al., 1986).

Clone A contained a 755-bp insert that corresponded in sequence to positions 46 to 795 of the S_2 cDNA. Only one sequence difference was observed: an extra adenosine base found in a series of 12 adenosine bases between positions 243 and 255 (Figure 6). This resulted in a frameshift that immediately introduced a stop codon into the translated sequence. In the PCR clones mentioned above that were derived from RNA of S_2S_2 anthers, six of the eight single-base mismatches occurred in this stretch of adenosines. Clone B contained two different inserts: a 481-bp fragment with no homology to the S_2 cDNA and a 700-bp fragment identical in sequence to the S_2 cDNA between nucleotides 69 and 767. The polyadenylation sites of the two clones were different and did not correspond to any other clones for S_2 cDNA previously characterized in this laboratory. Clone A had six terminal adenosine bases starting at nucleotide 794 relative to the S_2 cDNA sequence, whereas clone B contained only three terminal adenosine bases starting at nucleotide 765.

Detection of an S-RNase-Related Protein in Mature Pollen by Immunocytochemistry

Sections of hydrated mature pollen from plants homozygous for the S_2 allele were incubated with antibodies raised to either a peptide derived from a sequence unique to the S_2-RNase or to the corresponding region unique to the S_6-RNase. In protein gel blots, these two antibodies discriminate between purified S_2- and S_6-RNases. Figure 7 shows electron micrographs of hydrated pollen sections in which labeling by the anti–S-RNase antibodies was detected by a biotinylated anti-sheep antibody and streptavidin coupled to 15-nm gold particles. The anti-S_2 antibody (Figure 7A) bound to the inner part of the intine of the S_2 pollen grains, whereas the anti-S_6 antibody (Figure 7B) did not bind to the pollen. No binding of nonimmune serum was detected (data not shown). Binding of an anti-arabinosyl monoclonal antibody to the inner part of the intine was also detected (Figure 7C), and this region is also specifically stained by phosphotungstic acid (Figure 7D).

We also examined binding of the anti–S-RNase antibodies to anther sections from plants of both S_2S_2 and S_6S_6 genotypes (data not shown). Allele-specific labeling of the intine was detected only in developing pollen grains containing large vacuoles seen in stage 1 anthers; no binding to pollen in stage 2 or 3 anthers was detected, and no labeling of prevacuolate microspores was detected. However, some nonspecific labeling of the tapetal cell walls was detected at all stages with both the anti-S_2 and anti-S_6 antibodies.

DISCUSSION

The experiments reported here address the question: Could the style-expressed S-RNase of *N. alata* also represent the pollen component of the *S* locus? The alternative hypothesis—that a separate gene encodes the pollen function—has recently been favored because of the lack of detectable S-RNase expression in pollen or pollen tubes. We were encouraged to look for hybridization at an early stage of anther development by the unexpected finding that in *N. tabacum* transformed with the S_2-RNase cDNA under the control of the cauliflower

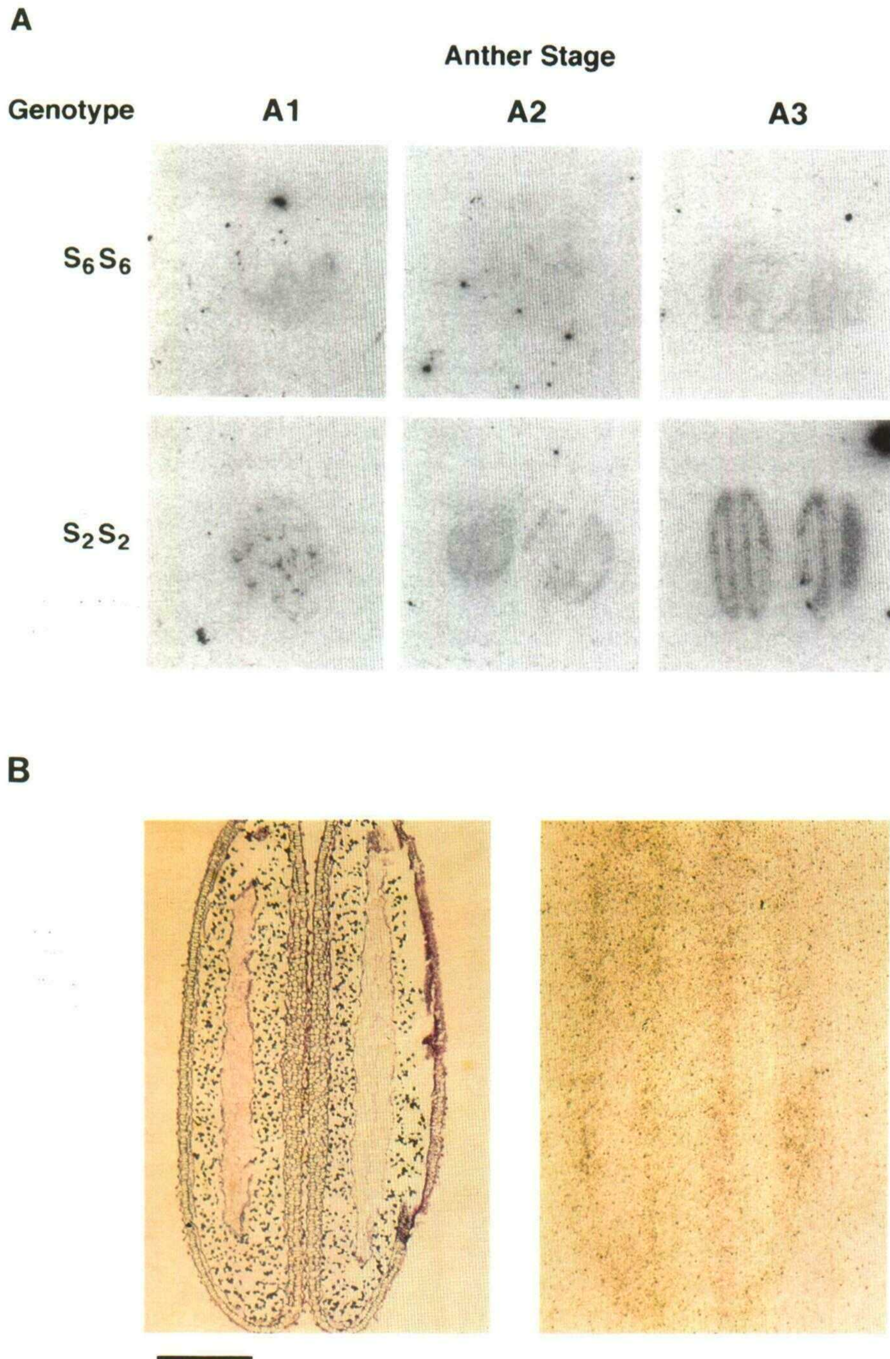

Figure 5. In Situ Hybridization of an S_2-RNase–Derived PCR Fragment to Sections of Developing Anthers from Buds of Genotypes S_2S_2 and S_6S_6.

(A) Anthers from 10-, 25- and 50-mm buds (A1, A2, and A3) were sectioned and hybridized to a ^{32}P-labeled pC2/pC3 PCR fragment produced from an S_2 cDNA template. Slides were exposed to Cronex MRF 31 x-ray film (Du Pont) for 5 days.

(B) Comparison of the in situ hybridization pattern obtained using the S_2-RNase–derived PCR fragment to probe a section of a stage 3 (50-mm bud) anther (genotype S_2S_2, right), and a light micrograph of the following section of the same anther stained with toluidine blue (left).

Bar = 0.5 mm.

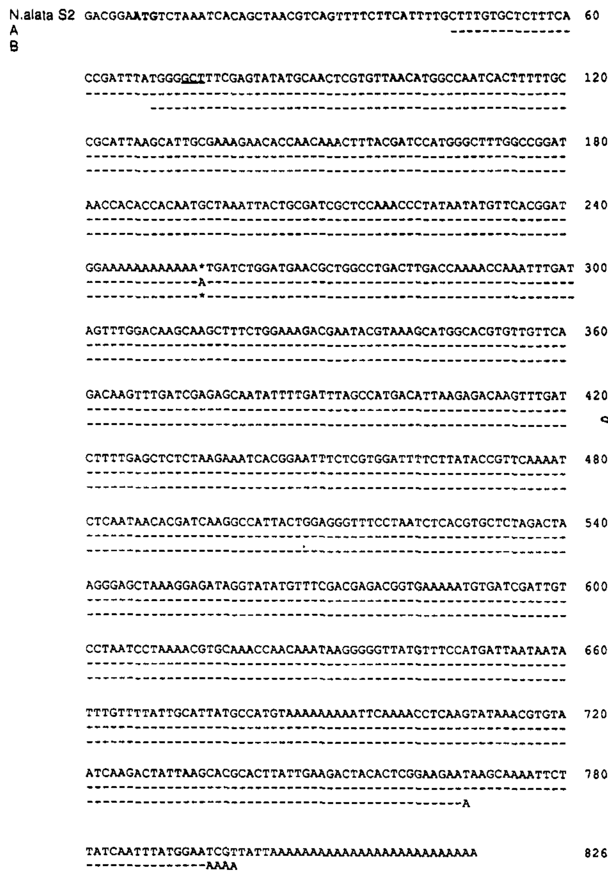

Figure 6. Nucleotide Sequence Comparison of Two cDNA Clones to the Published S_2 cDNA Sequence.

The full sequence of the S_2 cDNA (*N. alata* S2) is shown aligned with the sequences of two cDNA clones (A and B) isolated from a stage 3 anther cDNA library. Identical nucleotides are indicated by a dash and inserted gaps by an asterisk. Nonidentical nucleotides are indicated by the corresponding single-letter code. The start (ATG) codon of the S_2 sequence is shown in bold type, and the first codon of the mature protein is underlined. GenBank accession numbers are L25929 for clone A and L25930 for clone B.

mosaic virus 35S promoter, there was a higher level of expression in immature anthers than in mature anthers or pollen (Murfett et al., 1992). This suggested that the exons of the gene included signals directing expression in developing anthers.

A Transcript Homologous to the S-RNase Is Expressed in an Allele-Specific Manner in Developing Anthers of *N. alata*

We detected a transcript homologous to the S_2-RNase cDNA in RNA from S_2S_2 anthers at stages 2 (20- to 30-mm buds) and 3 (50- to 70-mm buds) by RNA gel blot analysis (Figure 2). Although the transcript hybridized to an S_2 probe, it was not detected using an S_6 probe, indicating that this message was specific to the S_2 allele. We also used an S_6 probe to detect a message in S_6S_6 anthers at the same developmental stages. This message was not detected by an S_2 probe (P. Dodds, unpublished results). No expression was detected in either mature pollen or pollen tubes, confirming previous reports of a lack of detectable S-RNase message in these cells.

The transcript for S-RNase in anthers is slightly smaller in size than the style message and is present at much lower levels. The size difference may be the result of differential polyadenylation that causes differences between S_2 transcripts in the style and stigma of *N. alata* (McClure et al., 1993). The poly(A) tail length has been implicated in mRNA stability (Mercer and Wake, 1985), and a short poly(A) tail on the pollen transcript could result in enhanced degradation of the message and account for the absence of detectable message for S-RNase in mature pollen.

The S-RNase transcript can also be detected in anthers at stages 2 and 3 by PCR, using primers directed to sequences conserved between S-RNases. RNA from anthers of both S_2S_2 and S_6S_6 genotypes gave PCR products of the predicted size with the primers pC2 and pC3. These PCR products are S allele specific, as demonstrated by nested PCR with primer pairs specific to the S_2 or S_6 sequences. Furthermore, the sequences of the PCR products corresponded to the sequence of the same region in either the S_2- or S_6-RNase cDNAs, indicating that they are the products of the respective alleles of the S-RNase gene. PCR with anther RNA and primers derived from sequences of other conserved regions of S-RNases gave products of the expected size, demonstrating that the transcript in anthers had a similar structural organization to the transcript from style. The anomalous product detected in developing anthers and mature pollen with primers pC1 and pC3 (see Table 2) did not hybridize on DNA gel blots to a probe that would detect several S alleles of *N. alata.*

In contrast to the results from RNA gel blot analysis, some PCR experiments detected S-RNase mRNA in mature pollen. This result was not consistently obtained and may represent the presence of very low levels of the transcript beyond stage 3 of anther development that would only be detectable by PCR.

The S-RNase Transcript Is Expressed in Postmeiotic Microspores

Hybridization of S_2 and S_6 probes to anther sections of both S_2S_2 and S_6S_6 genotypes confirmed the temporal pattern of expression of the S-RNase in developing anthers and its allele specificity. Hybridizing message was detected primarily in anthers at stage 3, with weaker expression at stage 2. Importantly, the message was confined to the developing pollen grains; it did not occur in the sporophytic tissue of the anthers.

The S-RNase gene was expressed during a narrow window of anther development. No expression was detected in stage 1 anthers (5- to 10-mm buds) that contain pollen in the early

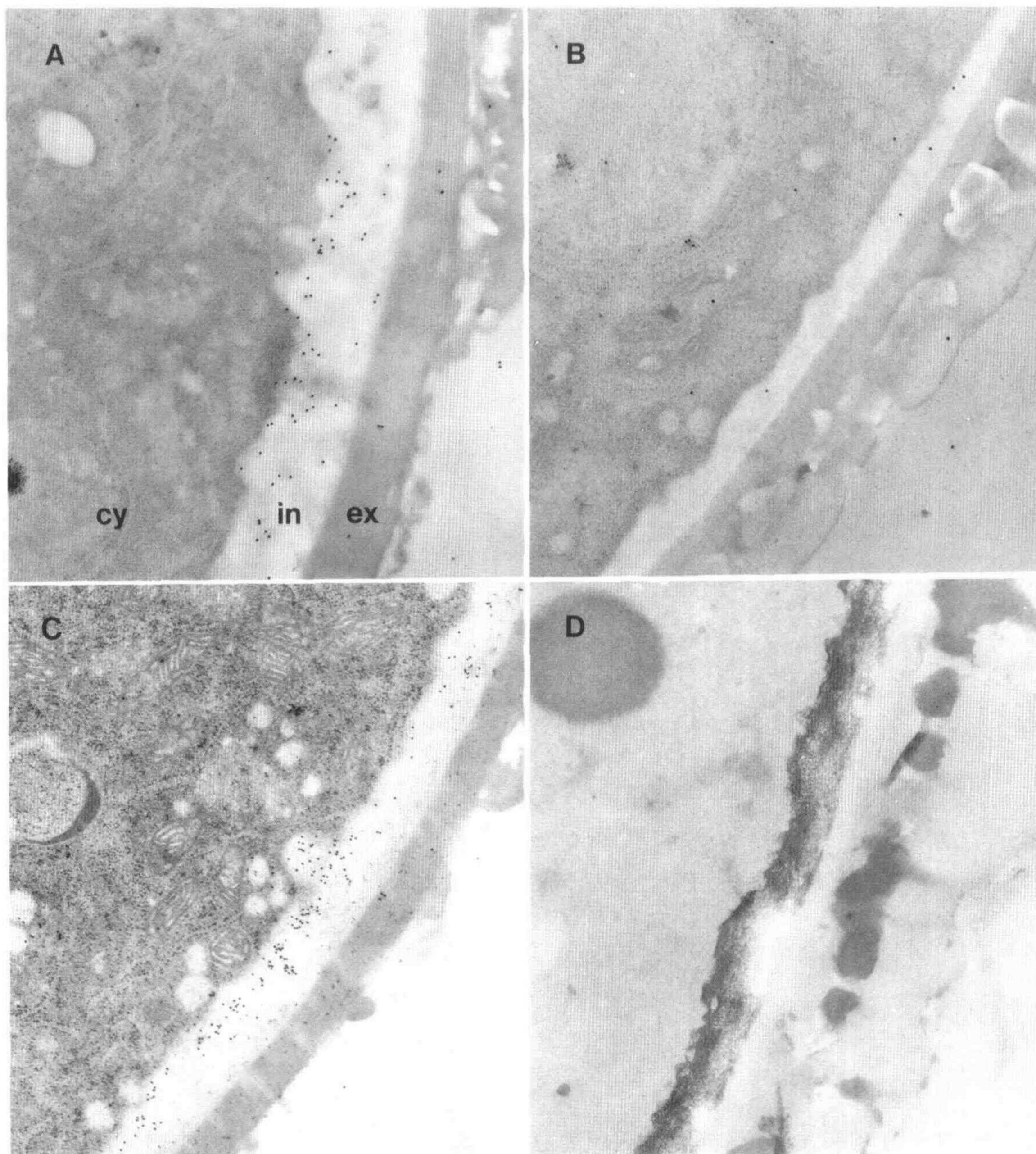

Figure 7. Immunolabeling of Hydrated Pollen Sections.

Pollen from an S_2S_2 parent was hydrated for 2.5 hr and then fixed and sectioned for immunolabeling and electron microscopy.

(A) Binding of an anti-S_2-RNase antibody to a pollen section. The primary sheep antibody was detected by biotinylated goat anti-sheep antibody followed by streptavidin conjugated to 15-nm colloidal gold. The exine (ex), intine (in), and cytoplasm (cy) are indicated.

(B) Binding of an anti-S_6-RNase antibody to a pollen section. Bound antibody was detected as is given for **(A)**.

(C) Binding of an anti-arabinosyl monoclonal antibody Fab fragment to a pollen section. Bound antibody was detected by a rabbit anti-mouse antibody conjugated to 15-nm colloidal gold.

(D) Pollen section stained with phosphotungstic acid.

Bar = 500 nm.

stages of development, from late meiosis through tetrad formation and breakdown to the early vacuolate stage (see Mascarenhas, 1989 for a detailed description of pollen development). The tapetum is very active at this stage, as reflected in the intense staining of the tapetal cells with acridine orange. Expression of the *S*-RNase was first detected in stage 2 anthers (20- to 30-mm buds) that contained vacuolate microspores which had completed meiosis. We had previously observed that microspore mitosis occurred at this stage (I. Bönig, unpublished results), so both uninucleate and binucleate microspores were present. These microspores contained little RNA: the premeiotic RNA had been degraded and the gametophytic RNA was beginning to be synthesized. The tapetum was poorly stained at this stage, indicating a reduced RNA content and lower level of metabolic activity. The window of expression continued to stage 3 anthers (50- to 70-mm buds), when the pollen grains were approaching maturity. At this stage, the developing pollen grains were rich in RNA that had been synthesized postmeiotically. Much of this RNA is stored in the mature pollen grains and is mobilized when the pollen germinates (Mascarenhas, 1989). The *S*-RNase message, however, was not detectable in the mature pollen grain.

The finding of *S*-RNase gene expression in microspores during postmeiotic development is consistent with a recent report (Thompson and Kirch, 1992) that a potato *S*-RNase promoter directed the expression of a β-glucuronidase (*GUS*) fusion gene in haploid pollen but not the tapetum of transgenic *N. tabacum* plants. Interestingly, Thorsness et al. (1991), using a *GUS* reporter gene fused to the promoter of an *S*-linked glycoprotein (*SLG*) gene (the *S* gene of the sporophytic SI system) from kale, observed a pattern of expression in the pistils of transformed *N. tabacum* similar to that observed for the *S*-RNase in *N. alata*. The *GUS* reporter gene was expressed gametophytically in binucleate and uninucleate microspores, a pattern similar to that now found for the *S*-RNase gene in developing pollen. These observations raise the possibility of evolutionary relatedness between the expression signals of the sporophytic and gametophytic SI systems.

The Transcript Is the Product of the *S*-RNase Gene

The RNA gel blot, PCR, and in situ hybridization experiments demonstrated that a transcript expressed in developing pollen of *N. alata* is homologous to the *S*-RNase gene. After screening 1.9×10^5 plaques, we isolated two cDNA clones from a stage 3 anther library (genotype S_2S_2) using an S_2-specific probe. For comparison, the abundance of this message in style-derived cDNA libraries is ~1.6% (Anderson et al., 1989). The sequence identity of the two anther-derived cDNAs to the published S_2 cDNA sequence (Figure 6) indicates that the transcript we have detected is the product of the *S*-RNase gene. We do not know if the presence of an extra adenosine in a series of 12 adenosines in the sequence of one of the clones was an artifact or represented heterogeneity in the message population. Interestingly, differences were also observed in the number of adenosine residues at this site in clones derived from PCR products of S_2S_2 anther RNA. However, the differences observed were not identical in each case, indicating that the changes were most likely experimental artifacts.

Although we cannot exclude the possibility that these cDNAs were derived from stylar RNA, several independent lines of evidence indicate that they come from anther RNA. We have shown by RNA gel blot analysis and in situ hybridization that there is a transcript present in developing pollen that hybridizes to the S_2 cDNA probe (Figures 2 and 5). In the preparation of the cDNA library, we took precautions to minimize the possibility of contamination. During collection of the anthers, we took care that there was no contaminating pistil material, and the cDNA library was prepared commercially in a laboratory free from S_2-RNase cDNAs. Under these conditions, we had a high degree of confidence that the library represented only anther RNA. Moreover, the two clones sequenced differed from all S_2 cDNA clones previously isolated in this laboratory in both their 5′ ends and their 3′ polyadenylation sites. This variation in polyadenylation sites is common in plant transcripts in general (Dean et al., 1986) and in the *S*-RNase transcripts in particular. Of the 10 S_2 cDNAs previously cloned from style and stigma of *N. alata*, all have different polyadenylation sites (Anderson et al., 1986; H. Du, unpublished results).

An *S*-RNase-Related Protein Is Present in the Intine of Hydrated Pollen

The lack of detectable levels of the *S*-RNase transcript in dry and germinated pollen and pollen tubes suggests that, if it has a role in SI, the *S*-RNase protein must be synthesized prior to the release of the mature pollen grain at anthesis. Attempts to detect proteins in mature pollen by protein gel blotting using *S*-RNase antibodies have been unsuccessful (S.-L. Mau, W. Jahnen, and A.E. Clarke, unpublished results), possibly due to the low abundance or difficulty of extraction of *S*-RNases from this tissue. However, using immunocytochemistry, we detected labeling by an S_2-specific antibody in the intine region of hydrated S_2 pollen (Figure 7). This labeling was not detected with an S_6-specific antibody. The region within the intine to which the S_2-specific antibody bound appeared to be structurally distinct, because it also bound an anti-arabinosyl antibody and was stained by phosphotungstic acid.

Immunolabeling of developing anthers produced some unexpected results. We found allele-specific binding of *S*-RNase antibodies to the inner wall (intine) of microspores at the earliest stage of postmeiotic development (the early vacuolate stage). Postmeiotic microspores formed a small subpopulation of the total microsporocytes at early stages of anther development, and the lack of detectable *S*-RNase transcript in stage 1 anthers may reflect the low abundance of these cells in the anther as a whole.

Despite repeated attempts, we found no binding to microspores or pollen grains at later stages of development that corresponded to stages at which the *S*-RNase mRNA was detected. This finding has led to the hypothesis that the *S*-RNase gene is expressed very early in postmeiotic pollen development and the product is laid down in the intine as it is formed. At this early stage, the *S*-RNase protein is detectable by immunocytochemistry; however, at later stages, although expression of the *S*-RNase gene continues, the *S*-RNase is not detectable by immunocytochemistry. We suggest that the protein is masked in some way, perhaps by the biosynthesis and deposition of other wall components. The protein would then remain present in the intine of mature pollen, and when the pollen grain germinates and turnover of the intine commences, the protein would be predicted to become accessible for antibody-based detection. This hypothesis is supported by the observation that the labeling of the intine of S_2 pollen grains by the anti-S_2 antibody was restricted to pollen that had germinated during the hydration period. Pollen grains that failed to germinate bound the antibody weakly (I. Bönig, unpublished observation).

The intine of germinated *N. alata* pollen is continuous with the wall of the emerging pollen tube (Rae, 1988), and the *S*-RNase expressed in pollen would be in a position in which it could come into contact with stylar *S*-RNases soon after germination. This model predicts recognition of incompatible pollen very early in pollen tube growth. Indeed, Heslop-Harrison et al. (1973) suggested that gametophytic SI is mediated by proteins contained in the intine that are products of the male gametophyte. Also, Linskens (1975) proposed that the recognition response in petunia occurs during the first few minutes of pollen–stigma contact, some time before the inhibition of pollen tube growth is measurable. Herrero and Dickinson (1981) reported differences in the rate of growth of incompatible and compatible pollen tubes within 2 to 3 hr of pollination of petunia styles. Thus, the localization of the *S*-RNase protein to the intine is consistent with, but not definitive evidence for, a role of the protein in determining the SI phenotype of pollen.

Implications for the Understanding of Gametophytic SI

Work on the genetics of gametophytic SI has revealed two important features. First, it is controlled by a single locus (*S* locus) with many alleles, and second, the *S* locus is expressed in both the diploid tissues of the pistil and in haploid pollen grains. Previously, it was shown that the expression of *S*-RNase, a product of the *S* locus of *N. alata*, was associated with the onset of SI in the style (Anderson et al., 1986). In this study, we demonstrated that the *S*-RNase gene is also expressed at low levels during postmeiotic pollen development and that the *S*-RNase protein is present in the intine of mature pollen, a location that would allow it to interact with components of the pistil. These features are consistent with, but not proof of, the involvement of *S*-RNase in determining the *S* phenotype of pollen and with a model for SI in which the interaction of identical *S* locus products in pollen and style leads to the arrest of pollen tube growth (Lewis, 1960). Whether such an interaction occurs and what is the molecular nature of the interaction are two questions that remain unanswered by these observations.

Although our data are consistent with a single-gene model, mutational studies of the *S* locus in a number of species indicate that expression of the *S* locus in pollen can be disrupted independent of expression in the style (Lewis, 1949, 1960; Pandey, 1965). This result has led to the suggestion that the *S* locus consists of two tightly linked genes: a style-specific gene (believed to encode the *S*-RNase in solanaceous SI species) and a pollen-specific gene (Lewis, 1949; Thompson et al., 1991). The nature of these pollen-part mutations is unclear; however, it has been postulated that they result from a duplication of the *S* locus that leads to a loss of expression in pollen for each copy (Pandey, 1965; de Nettancourt, 1977). Alternatively, pollen-part mutations may reflect events at other unlinked loci (modifier loci) involved in SI (Chetelat and DeVerna, 1991; Thompson et al., 1991). Further analysis of pollen-part mutants will be required before we can understand the role of *S*-RNase, if any, in determining the *S* phenotype of pollen. The finding that expression of the *S*-RNase also occurs in and is apparently restricted to developing pollen nonetheless points to some physiological function.

METHODS

Plant Materials

Tobacco (*Nicotiana alata*) plants of defined genotypes were maintained as previously described (Anderson et al., 1986). Organs used for RNA extraction were frozen in liquid nitrogen and stored at −70°C until use. Leaves were briefly ground and picked free of large veins before processing. Mature pollen was harvested by vortexing fresh, fully open anthers in a 1.5-mL centrifuge tube and then discarding the empty anthers. To obtain actively growing *N. alata* pollen tubes, *N. tabacum* (cv Wisconsin 38) flowers were emasculated 1 or 2 days prior to flower opening. At floral maturity, the stigmas were heavily pollinated with pollen from *N. alata*. After 24 hr, the *N. tabacum* pistils were removed and the stigmas dissected off. The pollen tubes were then teased out through a slit in the style.

Microscopy

Anther sections were stained with acridine orange by the method of Hafen et al. (1983) and examined by fluorescence microscopy using appropriate filters. Sections stained with toluidine blue (0.5% [w/v] in H_2O) were examined by light microscopy.

RNA Extraction and Blotting

RNA was prepared from organs of *N. alata* as previously described (McClure et al., 1990). RNA samples were separated on agarose gels

and transferred to Hybond N nylon membranes (Amersham). DNA probes were labeled with ^{32}P-dCTP. Prehybridization and hybridization were performed in 1.5 × SSPE (1 × SSPE is 0.15 mM NaCl, 10 mM Na_3PO_4, 1 mM EDTA), 1% SDS, 4 mg/mL denatured herring sperm DNA, 1% Blotto at 68°C, and final washes were done at 68°C in 1 × SSPE, 0.5% SDS.

cDNA Synthesis and Polymerase Chain Reaction Analysis

First-strand cDNA synthesis and polymerase chain reaction (PCR) amplification utilized the following primers:

pC1 5'-CAACTCGTGTTAACATGGCCA
pC2 5'-AACTTTACGATCCATGGACTTTGGC
pC2R 5'-GCCAAAGTCCATGGATCGTAAAGTT
pC3 5'-ACAACACGTGCCATGCTT
pC5 5'-AAACATATGCCTACCTCC
pV1.6 5'-GGTAAAGAAGATGACTATAACAT
pV1.2 5'-TTACTGCGATCGCTCCAAACC
pV2.6 5'-TTCATACAATCAGCTTTCTCTC
pV2.2 5'-TCCAAACTATCAAATTTGGTTT

Oligonucleotide synthesis was performed on a DNA synthesizer (model 391; Applied Biosystems, Foster City, CA).

First-strand cDNA was synthesized by avian myeloblastosis virus reverse transcriptase (0.5 units per µL; Promega) in 20 µL of PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.05% Tween 20, 0.01% gelatin) with 1.0 mM of deoxynucleotide triphosphates and 1 unit per µL of RNasin. Total RNA (1 µg) was used with 0.1 µg of oligo(dT) primer or 5 µg of total RNA with 50 pmol of the primers pC5, pC3, or pC2R. The reaction mixture was incubated at 42°C for 1 hr. Five microliters of the cDNA reaction mix was used for PCR.

PCRs were performed using Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT) in 100 µL of PCR buffer with 20 µM deoxynucleotide triphosphates and 40 pmol of each primer. A thermocycler (Perkin-Elmer-Cetus) was used to perform 40 cycles of the following thermal profile: 95°C, 30 sec; 55°C, 1 min; 72°C, 30 sec; this was followed by a final 10-min extension at 72°C.

For cloning and sequencing of PCR products, the vector pBluescript KS+ (5 µg; Stratagene) was cut with EcoRV and incubated with Taq polymerase and dTTP (2 mM) in a 20-µL volume of PCR buffer. One microliter (250 ng) of the T-tailed vector was ligated to the PCR products, and the ligations were transformed into *Escherichia coli* XL1-Blue. Recombinant bacteria were chosen, and plasmids were analyzed by restriction digest for the presence of inserts of the correct size. Inserts were sequenced using a cycle sequencing kit (Taq DyeDeoxy; Applied Biosystems) and analyzed on a DNA sequencer (model 373A; Applied Biosystems).

cDNA Library Construction and Screening

Poly(A)$^+$ RNA was purified from total RNA of anthers from 50- to 70-mm buds (genotype S_2S_2) by two rounds of oligo(dT) cellulose chromatography using an mRNA purification kit (Pharmacia, Uppsala, Sweden). A cDNA library was constructed in λZAPII (Stratagene) from 1.0 mg of poly(A)$^+$ RNA (Clonetech Laboratories, Palo Alto, CA), using both random- and oligo(dT)-primed cDNAs. The titer of the unamplified library was 1.8 × 10^6 plaque-forming units. Nylon filters containing 2.0 × 10^5 plaques were hybridized with ^{32}P-dCTP–labeled DNA as given above.

In Situ Hybridization

Anther sections were prepared using a sectioning aid (Instrumedics, Teaneck, NJ) and hybridized as described by Anderson et al. (1986). The ^{32}P-labeled DNA probes were hybridized to the sections for 16 hr at 42°C. Slides were exposed to Cronex MRF 31 x-ray film (Du Pont) for 5 days.

Electron Microscopy and Immunocytochemistry

Mature pollen was hydrated in 2% sucrose, 50 mM boric acid for 2.5 hr and fixed according to the method of Gray et al. (1991), except that Pipes was used at 30 mM. The pollen were embedded in Spurr's resin (Spurr, 1969). For immunolabeling, 90-nm sections were cut on an ultramicrotome, collected on gold grids, and etched for 20 min in H_2O_2 according to the method of Nishizawa et al. (1990). Treated sections were blocked with 0.1% ovalbumin (Sigma) in phosphate-buffered saline, 0.5 M NaCl for 30 min and then incubated with affinity-purified sheep anti–S_2-RNase (2.8 µg per mL; Anderson et al., 1989) or anti–S_6-RNase (2.8 µg per mL; Murfett, 1991) antibodies for 1 hr. After washing with phosphate-buffered saline, 0.2% Tween 20, the bound antibodies were detected by a biotinylated goat anti–sheep antibody (Amersham) and followed by streptavidin conjugated to 15-nm colloidal gold (Sigma). Nonimmune sheep serum (Miles Laboratories, Elkhart, IN) was used as a control. Labeling with an anti-arabinosyl monoclonal antibody Fab fragment was performed according to the method of Anderson et al. (1987). All sections were counterstained with uranyl acetate (5% aqueous solution). Unlabeled sections were stained with phosphotungstic acid according to the method of Roland (1978). For immunolabeling of anther sections, anthers were fixed, embedded in London Resins gold, and labeled with the anti-S_2 and anti-S_6 antibodies according to the method of Gray et al. (1991). All sections were examined in an electron microscope (model 1200; Jeol, Tokyo, Japan).

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