The Plant Cell, Vol. 3, 271–283, March 1991

Action of the Style Product of the Self-Incompatibility Gene of *Nicotiana alata* (*S*-RNase) on in Vitro-Grown Pollen Tubes

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The products of the *S*-locus expressed in female tissues of *Nicotiana alata* are ribonucleases (*S*-RNases). The arrest of growth of incompatible pollen tubes in styles may result from entry of the *S*-RNase into the pollen tube and degradation of pollen tube RNA. We investigated the action of isolated *S*-RNases on pollen tubes grown in vitro and found that *S*-RNase is taken up by the pollen without substantial alteration. The *S*-RNases inhibit incorporation of exogenously added radioactive amino acids into protein by the germinated pollen. The *S*-RNases also inhibit in vitro translation of pollen tube RNA in a wheat germ cell-free extract. We found no evidence for a specific mRNA substrate for the *S*-RNases, which implies that if RNase activity is involved in the control of self-incompatibility, allelic specificity is more likely to depend on the selective uptake of *S*-RNases into pollen tubes or their selective activation or inactivation by pollen factors, rather than cleavage of a specific substrate. Heat treating S_2-RNase largely destroys its RNase activity but increases its inhibitory effect on in vitro pollen tube growth. This effect is not due to an increased uptake of S_2-RNase by the pollen but is associated with a greatly enhanced accumulation of S_2-RNase on the outer surface of the pollen grains.

INTRODUCTION

The most widespread mechanism of self-incompatibility in flowering plants is gametophytic self-incompatibility, which in many cases is controlled by a single polyallelic genetic locus (de Nettancourt, 1977). Growth of a pollen tube bearing one of the two self-incompatibility alleles (*S*-alleles) carried by the recipient pistil is inhibited in the upper region of the style.

A number of *S*-locus products have been identified in the female tissues of several members of the Solanaceae (Bredemeijer and Blass, 1981; Anderson et al., 1986; Kamboj and Jackson, 1986; Mau et al., 1986; Jahnen et al., 1989a; Clark et al., 1990; Kirch et al., 1990; Xu et al., 1990) and corresponding cDNA clones obtained and sequenced (Anderson et al., 1986, 1989; Ai et al., 1990; Clark et al., 1990; Kheyr-Pour et al., 1990). These *S*-locus products are glycosylated, basic glycoproteins that accumulate along the path that the pollen tubes follow to reach the ovule; in particular, they are secreted by the cells of the style-transmitting tract and the inner epidermis of the placenta (Cornish et al., 1987). In recent studies of catalytically active regions of RNases, it was noticed that potential active site histidine residues, as well as several cysteine residues in RNase T_2 (Kawata et al., 1988) and RNase Rh (Horiuchi et al., 1988), are homologous with the S_2-glycoprotein of *Nicotiana alata*. Further studies have provided direct evidence that the *N. alata* S_1-, S_2-, S_3-, S_6-, and S_7-glycoproteins are indeed RNases (McClure et al., 1989). Sequence analysis predicts that other *S*-glycoproteins from solanaceous plants will also have RNase activity (Haring et al., 1990), and this has been demonstrated for *S*-glycoproteins of petunia (Broothaerts and Vendrig, 1990; Clark et al., 1990).

Based on these results, we have suggested that solanaceous *S*-glycoproteins (*S*-RNases) are allele-specific cytotoxins (McClure et al., 1989). The observation that pollen RNA is degraded in incompatible matings but not compatible matings is consistent with this suggestion (McClure et al., 1990), but it is not yet clear whether this is the primary cause of growth inhibition in incompatible pollen tubes. To test the hypothesis that *S*-RNases attack the protein biosynthetic apparatus of incompatible pollen tubes in an *S*-allele-specific manner, we have examined the uptake of *S*-RNases and their effect on protein synthesis by in vitro-grown pollen tubes. In this report, we show that *S*-RNases can gain access to the cytoplasmic compartment of the pollen tube and are effective inhibitors of translation in both cell-free extracts and in pollen tubes.

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RESULTS

Uptake of S-RNase by in Vitro-Grown Pollen Tubes

Pollen (genotypes S_2, S_3, and S_6) was incubated in vitro in the presence of tritiated S-glycoprotein ([^{3}H]S_2-RNase) for 3 hr and prepared for light microscopy, and the location of radioactive material determined was by autoradiography. As shown in Figure 1A, the radioactive material was present within the cytoplasm and cell walls of the S_2-pollen grains. It was present to a lesser extent within the cytoplasm of pollen tubes, and labeled material was also associated with the outer surface of the tubes, as shown in Figure 1B. A similar pattern of distribution was observed when S_3-pollen (shown in Figure 1C) and S_6-pollen (not shown) were separately incubated with the [^{3}H]S_2-RNase.

To resolve the subcellular localization of the S_2-RNase, pollen grain and tube sections were examined by immunogold electron microscopy using an antibody raised to a

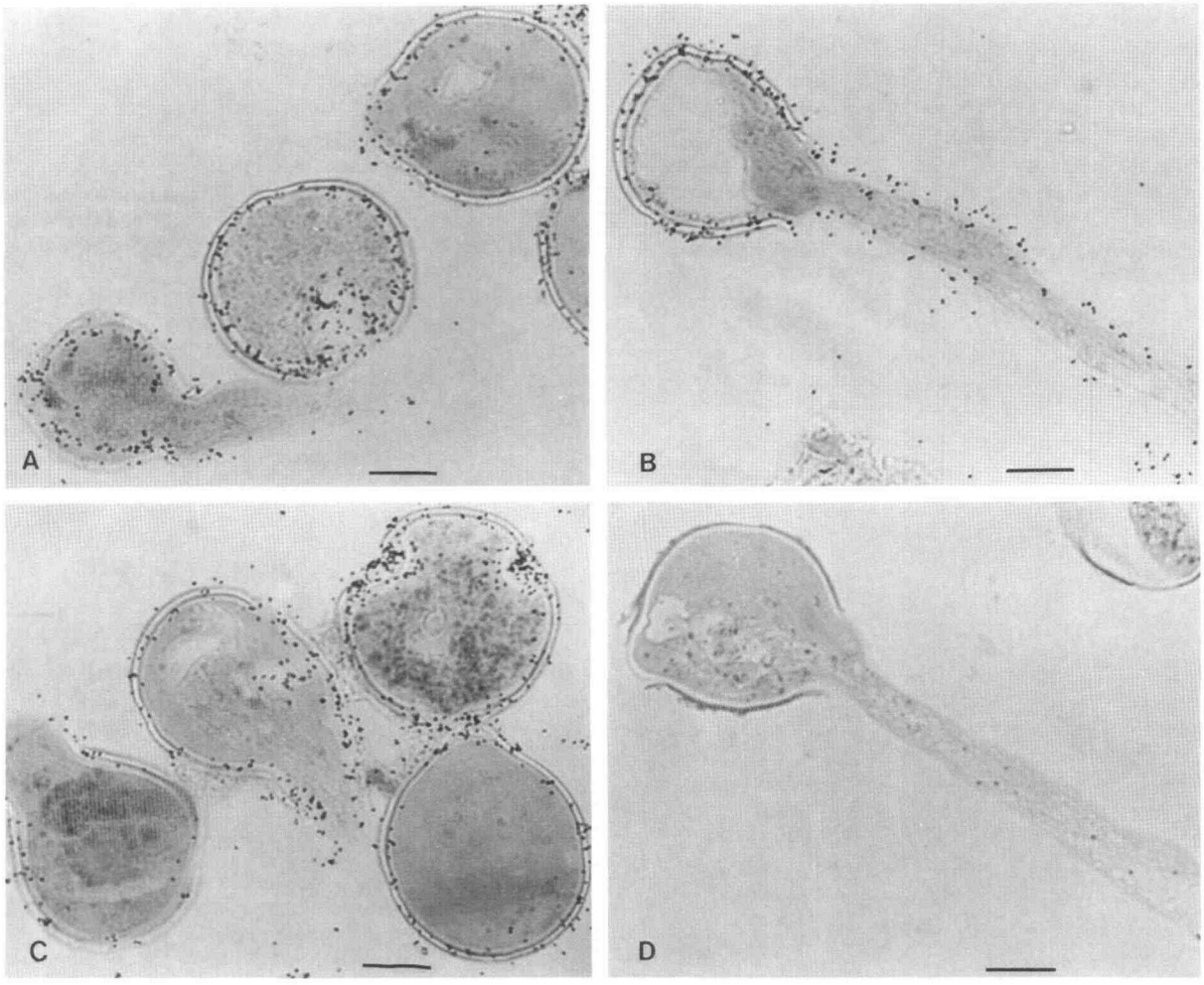

Figure 1. Uptake of [^{3}H]S_2-RNase into in Vitro-Grown Pollen Tubes.

(A) and **(B)** Sections through S_2-pollen tubes grown in the presence of [^{3}H]S_2-RNase. Radioactivity is located within the cytoplasm and cell wall of the pollen grains and on the outer surface of the pollen tubes.

(C) Section through S_3-pollen tubes grown in the presence of [^{3}H]S_2-RNase. Radioactivity is distributed in S_3-pollen in a pattern similar to that seen in S_2-pollen.

(D) Section through an S_2-pollen tube grown in unlabeled S_2-RNase. The darker spots are starch grains, which are not as distinct as the silver grains seen in **(A)**, **(B)**, and **(C)**.

Scale bars = 10 μm.

synthetic peptide corresponding to an allele-specific region of the S_2-RNase (S_2-antibody) (Anderson et al., 1989). In the germinated pollen grain, as shown in Figure 2A, the S_2-RNase was associated primarily with a specific layer of the intine close to the plasma membrane and also in the cytoplasm. In the pollen tube, close to the tip, Figure 2B shows gold label present within both the inner wall and the cytoplasm. A similar distribution was observed when S_3-pollen (shown in Figure 2C) or S_6-pollen (not shown) was incubated with S_2-RNase. There was apparently less gold labeling in sections of pollen of these genotypes. In the control experiment shown in Figure 2D, few gold particles bound to the sections (S_2-pollen) that were treated with S_2-antibody preincubated with S_2-peptide. A few gold particles, which were scattered throughout the sections, were observed on sections of pollen grown in the absence of S-RNase and incubated with the antibody (not shown).

Integrity of S_2-RNase after Incubation with Pollen

We performed experiments to determine whether the labeled material observed by autoradiography (Figure 1) was indeed intact S_2-RNase. Pollen (genotypes S_2, S_3, or S_6) was incubated for 3 hr in the presence of [^{3}H]S_2-RNase. Samples of the incubation media were examined by SDS-PAGE. Proteins associated with pollen tubes were extracted from rinsed pollen tubes by boiling in SDS-PAGE loading buffer and are shown separated on the same gel in Figure 3. The radioactive material recovered from the incubation media corresponded to authentic S_2-RNase (32 kD). There was no evidence of lower molecular weight products that might have arisen from degradation of S-RNase in the medium. Minor radioactive protein bands can be seen in samples that were heavily loaded (Figure 3, lanes 1 to 4) (see also Figure 6, lane 7). These accounted for less than 1% of the radioactivity and correspond to minor impurities in the original sample of isolated S_2-RNase. The radioactivity extracted from the rinsed pollen of the three genotypes also comigrated with the authentic S_2-RNase (Figure 3, lanes 5 to 7).

As an initial attempt to establish the nature of the S_2-RNase association with the pollen tubes, we examined the relative amounts of S_2-RNase taken into the pollen tubes or tightly bound to the tubes. Three methods were used for removal of S_2-RNase from the outer surface of the pollen tubes. Pollen tubes were either (1) rinsed extensively in fresh incubation medium, (2) rinsed in incubation medium and then incubated with proteinase K, or (3) rinsed in incubation medium and then in a medium containing 100 mM NaCl before extraction of the proteins. (Proteinase K digested isolated S_2-RNase, whereas trypsin and other proteases tested were not effective.) After 3 hr of growth, approximately 5% of the S_2-RNase added to S_2-pollen remained associated with pollen tubes after rinsing in a medium containing 100 mM NaCl. This is equivalent to approximately 18 μg/mg of pollen (weight before hydration). After treatment either with medium alone or medium with added proteinase K, there was a slightly higher percentage of associated S_2-RNase. These results are summarized in Table 1. Figure 4 shows that in each case the material recovered was a single component of 32 kD.

Association of Heat-Treated S-RNase with Pollen after Incubation in Vitro

S_2-RNase boiled for 15 min and cooled on ice had barely detectable RNase activity (average of five separate experiments gave less than 2% of original activity). RNase activity was not regained if heat-treated material was cooled slowly to room temperature or incubated for 3 hr in pollen growth medium. In spite of this low RNase activity, boiled [^{3}H]S_2-RNase (1.35 mg/mL) was an extremely effective inhibitor of growth of both S_2-pollen and S_6-pollen tubes. Pollen grains hydrated normally, and a few germinated to form short tubes (less than 25 μm in length compared with 100 μm to 200 μm for controls incubated with untreated S_2-RNase). Autoradiography showed an accumulation of heat-treated S-RNase on the outer surface of the S_2-pollen, in contrast to the pattern for untreated S_2-RNase, which accumulated mainly within the pollen grain walls, as shown in Figure 5. This pattern of accumulation was also seen in S_6-pollen exposed to heat-treated S_2-RNase (data not shown). To quantify the amount of S-RNase associated with S_2-pollen after incubation with heat-treated [^{3}H]S_2-RNase, pollen was rinsed with medium containing 100 mM NaCl, the proteins were extracted, and radioactivity associated with each fraction was determined. Approximately 22% of the radioactivity derived from the boiled [^{3}H]S_2-RNase remained associated with the pollen after rinsing in medium containing 100 mM NaCl. This is equivalent to approximately 80 μg/mg of pollen (weight before hydration). The radioactive material in each extract and that remaining in the incubation medium was 32 kD, as shown in Figure 6.

Estimation of the Amount of S_2-RNase Associated with the Soluble Fraction of Pollen Tubes

Pollen (genotype S_2) was incubated for 3 hr, separately with untreated or heat-treated [^{3}H]S_2-RNase (1.35 mg/mL), and washed in incubation medium containing 100 mM NaCl. An estimate of the amount of untreated or heat-treated [^{3}H]S_2-RNase remaining associated with the soluble cytoplasmic and wall fractions was obtained by disrupting the treated pollen and separating it into soluble and cell wall fractions after extracting with 0.1 M Tris-HCl, pH 6.8. The insoluble residue was re-extracted by boiling in SDS-PAGE loading buffer. Approximately 50% of the

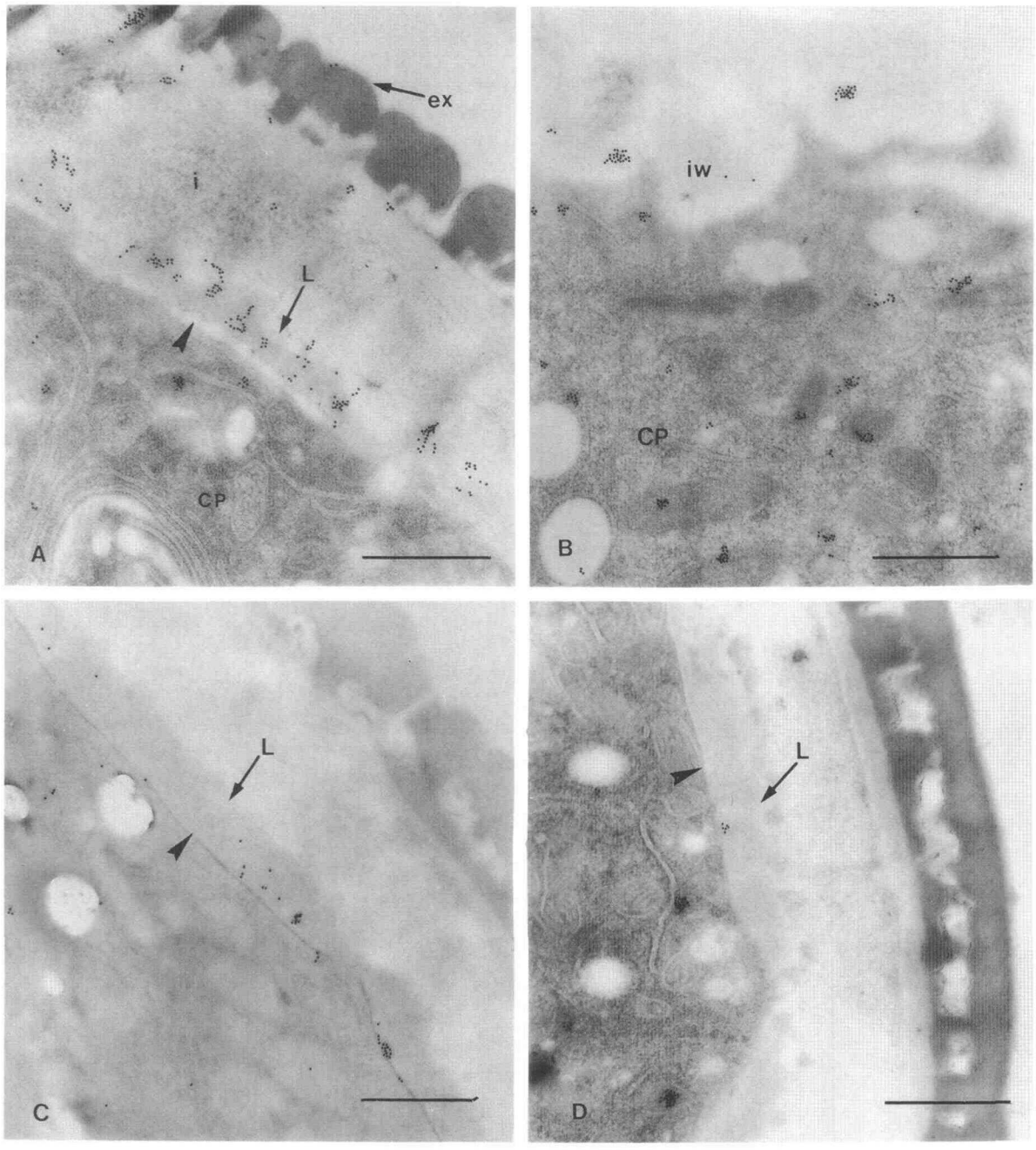

Figure 2. Electron Micrographs Showing Immunogold Localization of S_2-RNase in Pollen Tubes after Incubation (3 hr) in the Presence of S_2-RNase, Using Anti-S_2-Peptide Antibody.

(A) Section through a germinated S_2-pollen grain. Gold particles are located in a layer (L) of the intine (i) close to the plasma membrane (arrow). ex = exine, CP = cytoplasm.

(B) Section through an S_2-pollen tube close to the tip. Gold particles are in the cytoplasm (CP) and developing inner wall (iw).

(C) Section through an S_3-pollen grain after incubation (3 hr) in the presence of S_2-RNase. Gold particles are present in the same wall layer (L), close to the plasma membrane (arrow), as in S_2-pollen.

(D) Control. Section similar to **(A)** after preincubation of the S_2-peptide antibody with synthetic S_2-peptide. Gold particles are detected at a low frequency in the cytoplasm and rarely in the wall.

Scale bars = 1 μm.

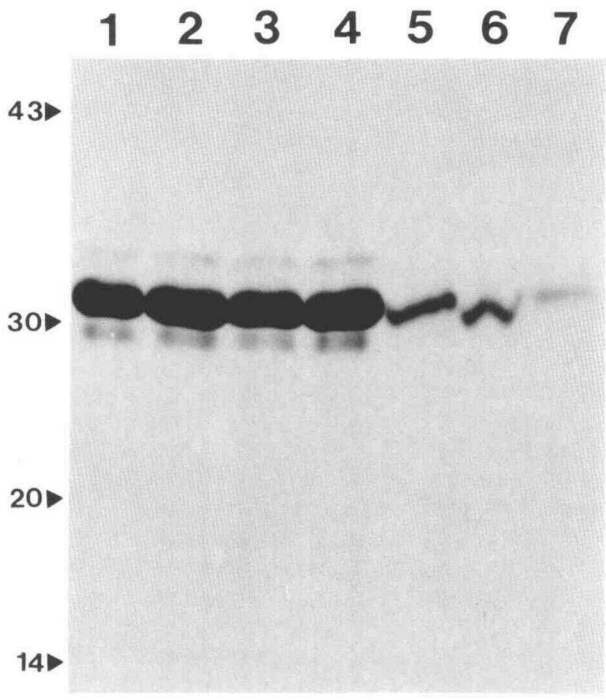

Figure 3. Integrity of S_2-RNase after Incubation with Pollen.

SDS-PAGE (15%) fluorograph. Lane 1, S_2-RNase incubated in medium with no added pollen. Lanes 2, 3, and 4, medium recovered from incubations of [^{3}H]S_2-RNase with S_2-pollen, S_3-pollen, and S_6-pollen, respectively. Lanes 5, 6, and 7, material extracted from pollen by boiling in SDS-PAGE loading buffer. S_2-pollen, S_3-pollen, and S_6-pollen tubes were rinsed twice after growth in the presence of [^{3}H]S_2-RNase. Samples loaded contained 5% of the medium (lanes 1, 2, 3, and 4) or 50% of the pollen tube extracts (lanes 5, 6, and 7) from 100-μL incubations. Positions of molecular weight markers are shown as $M_r \times 10^{-3}$. In each lane, the major component corresponded to the 32-kD S_2-RNase.

radioactivity recovered from pollen incubated with untreated [^{3}H]S_2-RNase was associated with the soluble fraction (two separate experiments yielded 60% and 41%); in contrast, less than 1% of the heat-treated [^{3}H]S_2-RNase was recovered in the soluble fraction by this method (Figure 6).

Effect of *S*-RNases on mRNA Translation in a Cell-Free Extract

We have shown recently that pollen rRNA is degraded after incompatible matings in *N. alata* and proposed that the pollen tube growth inhibition characteristic of self-incompatibility in the Solanaceae may be the result of an effect of *S*-RNases on protein biosynthesis (McClure et al., 1990). Therefore, we designed experiments to test the effect of *S*-RNase on protein biosynthesis directly. RNA extracted from in vitro-grown S_2-pollen tubes was translated in a wheat germ, cell-free extract in the presence or absence of S_2-RNase, S_6-RNase, or bovine pancreatic RNase A. Table 2 shows that at a concentration of 0.01 units/mL both S_2-RNases and S_6-RNases inhibited cell-free translation to approximately 70% of the control rate (no RNase added) and at 1 unit/mL and 10 units/mL incorporation was almost totally arrested. Equal concentrations (units/milliliter) of S_2-RNases and S_6-RNases inhibited translation of S_2-pollen tube RNA to approximately the same extent (Table 2). The extent of inhibition of translation by *S*-RNases was approximately the same as that by an equal concentration of RNase A. Figure 7 shows that the pattern of proteins produced after inhibition with all three RNases was similar, and translation of all pollen tube mRNAs was inhibited to a similar extent. There was no indication of preferential inhibition of synthesis of any specific protein by any of the RNases tested. Heat-treated *S*-RNase did not inhibit protein synthesis any more than an equal concentration (milligram/milliliter) of BSA.

Effect of *S*-RNases on the Synthesis of Proteins by in Vitro-Grown Pollen Tubes

Pollen (genotype S_2) was germinated and grown in vitro in the presence or absence of S_2-RNase, S_6-RNase, or RNase A. After 2.5 hr, aliquots were removed to estimate the length of the pollen tubes. Control pollen tubes grown in the absence of added RNase were approximately 200 μm to 300 μm in length. The length of pollen tubes grown in S_2-RNase (2 mg/mL) was estimated to be 50% of that of controls and when grown in S_6-RNase (2 mg/mL) was

Table 1. Amount of [^{3}H]S_2-RNase Remaining Associated with S_2-Pollen Tubes after 3 hr of Growth in Vitro in the Presence of [^{3}H]S_2-RNase

	Washing Conditions		
	Incubation Medium	Incubation Medium + Proteinase K (0.2 mg/mL)	Incubation Medium + NaCl (100 mM)
Expt. 1	5.6	5.3	3.6
Expt. 2	11.7	7.5	6.2
Mean	8.7	6.4	4.9

The germinated pollen grains were rinsed in fresh incubation medium, or incubation medium supplemented with proteinase K (0.2 mg/mL) for 15 min, or NaCl (100 mM). The figures are expressed as total counts recovered as a percentage of total counts added.

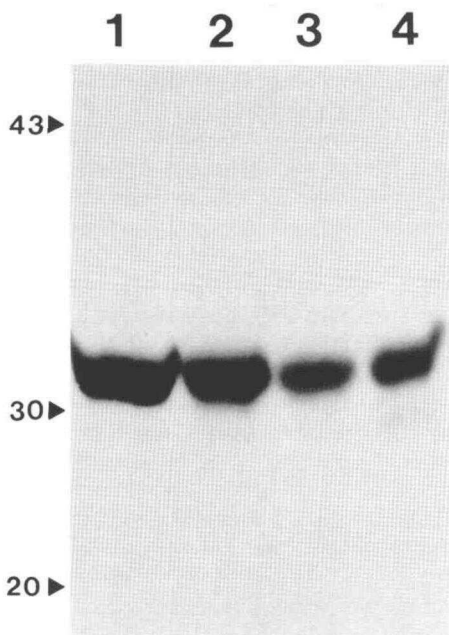

Figure 4. Association of S_2-RNase with Pollen Tubes after Protease Treatment or Rinsing in Medium Containing 100 mM NaCl.

SDS-PAGE (15%) fluorograph. Lane 1, S_2-RNase incubated in medium in the absence of pollen. Lanes 2, 3, and 4, proteins extracted from pollen tubes (genotype S_2) after incubation in S_2-RNase for 3 hr and rinsing in incubation medium (lane 2), incubation medium + 0.2 mg/mL proteinase K (lane 3), incubation medium + 100 mM NaCl (lane 4). Samples containing equal radioactivities were loaded. Positions of molecular weight markers are shown as $M_r \times 10^{-3}$.

estimated to be 75% of that of controls. The growth of pollen tubes in RNase A (0.3 mg/mL and 0.5 mg/mL) was also inhibited. Lower concentrations of RNase A were used because RNase A has a higher specific RNase activity than the *S*-RNases. After 30-min labeling with ^{35}S-methionine, pollen tube proteins were extracted (after a total of 3 hr of growth), and the radioactivity of the TCA precipitate was determined. Table 3 shows that treatment with any of the three RNases reduced the incorporation of ^{35}S-methionine into pollen tube proteins; the reduction was approximately proportional to the estimated lengths of pollen tubes. SDS-PAGE did not show any differences in the pattern of pollen tube proteins synthesized after treatment with the three RNases during the labeling period, as shown in Figure 8. When mixed, tritiated amino acids were used for labeling and similar relative levels of radioactivity were incorporated in the presence of these three RNases (data not shown).

DISCUSSION

The experiments reported here begin to address the question of whether the RNase activity of the products of the *S*-locus in the style, the *S*-glycoproteins (*S*-RNases), could be involved in the arrest of pollen tube growth by entering the pollen tube and degrading pollen RNA.

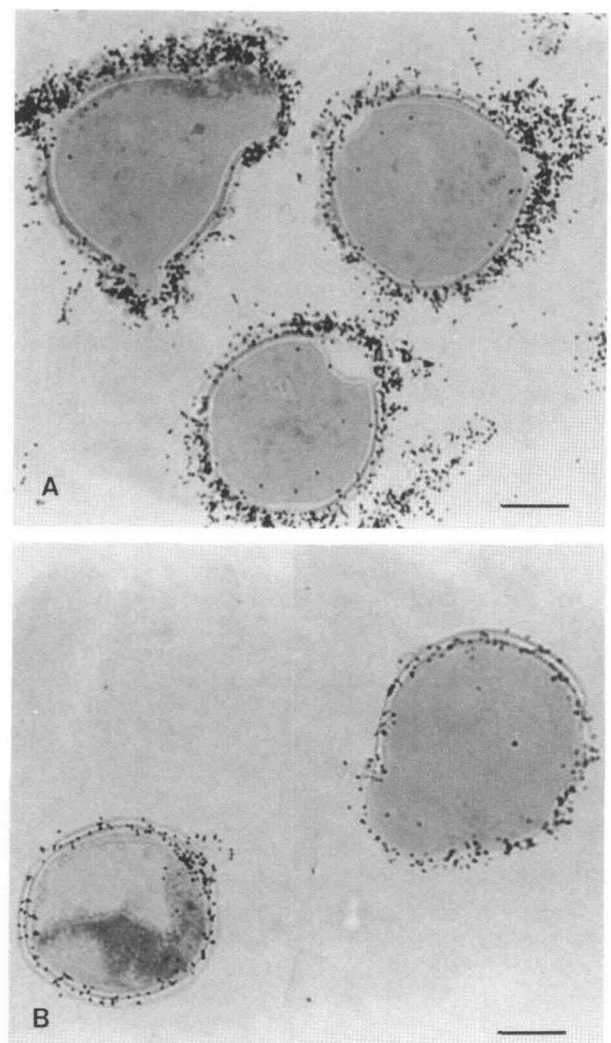

Figure 5. Association of Heat-Treated S_2-RNase with Pollen after Incubation.

Autoradiography of sections through S_2-pollen grains.
(A) After incubation in heat-treated [^{3}H]S_2-RNase.
(B) After incubation in untreated [^{3}H]S_2-RNase.
Heat-treated [^{3}H]S_2-RNase is detected predominantly around the outer surface of the pollen grains. With untreated [^{3}H]S_2-RNase, less total signal is observed and the majority is within the wall and cytoplasm of the pollen grain. Scale bar = 10 μm.

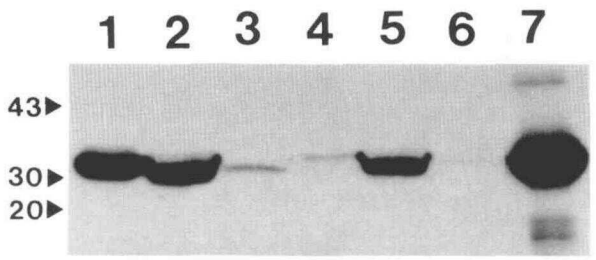

Figure 6. Comparison of Untreated or Heat-Treated *S*-RNase Associated with the Soluble Fraction of in Vitro-Grown Pollen Tubes.

Pollen (genotype S_2) was incubated (3 hr) and rinsed in incubation medium containing 100 mM NaCl, as described in Methods. Fluorograph of 15% SDS-PAGE. Lane 1, [^{3}H]S_2-RNase. Lane 2, incubation medium containing [^{3}H]S_2-RNase after incubation with S_2-pollen. Lane 3, proteins extracted from S_2-pollen tubes by fragmentation in 0.1 M Tris-HCl, pH 6.8, after incubation in the presence of [^{3}H]S_2-RNase. Lane 4, proteins extracted from insoluble material remaining after extraction described for lane 3 by boiling in SDS-PAGE loading buffer. Lane 5, growth medium containing heat-treated [^{3}H]S_2-RNase after incubation with S_2-pollen. Lane 6, proteins extracted from S_2-pollen tubes by fragmentation in 0.1 M Tris-HCl, pH 6.8, after incubation in the presence of heat-treated [^{3}H]S_2-RNase. Lane 7, proteins extracted from insoluble material remaining after extraction described for lane 6 by boiling in SDS-PAGE loading buffer. Positions of molecular weight markers are shown as $M_r \times 10^{-3}$.

S-RNases Are Taken Up by in Vitro-Grown Pollen Tubes

The *S*-RNases, when incubated with pollen tubes in vitro, enter the tubes. This is shown by direct microscope observations (Figures 1 and 2) and is supported by the inhibition of pollen tube protein biosynthesis, which implies entry of the *S*-RNases and action on the protein biosynthetic machinery (Table 3). Observations were made at the light microscope level by autoradiography after incubation of pollen with [^{3}H]S_2-RNase and at the ultrastructural level by immunoelectron microscopy using antiserum specific for the S_2-RNase. The experiments show that the S_2-RNase is present in both walls and cytoplasm of the pollen tubes.

The S_2-RNase was observed to be concentrated in a specific layer of the pollen grain intine close to the plasma membrane. This may simply reflect a particular stage in the passage of S_2-RNase from the extracellular medium to the cytoplasm, or this layer may play a direct role in the uptake mechanism. Resolution of these possibilities would require study of distribution of the *S*-RNase during a time course of incubation with pollen. There was no evidence of the *S*-RNase in the pollen cytoplasm being enclosed within membrane-bound vesicles or associated with particular organelles, implying that it could make contact with the ribosomes and protein synthetic machinery.

S_2-RNase was also detected inside S_3-pollen tubes (Figures 1C and 2C) and in the same layer of the intine as in S_2-pollen tubes (Figures 1A, 1B, 2A, and 2B). Similar results were obtained with S_6-pollen tubes (not shown). The micrographs of Figures 1 and 2 suggest that less S_2-RNase may be taken into S_3-pollen than into the S_2-pollen, but a statistical analysis of a larger sample of pollen would be required to confirm this point. These results are consistent with the observation that *N. alata S*-RNases inhibit in vitro growth of pollen of these *S*-genotypes with a low level of allelic specificity in vitro (Jahnen et al., 1989a).

The *S*-RNases were remarkably resistant to protease digestion. Pollen grains characteristically contain proteases in their outer wall (exine) that are released into the surrounding medium when the pollen is hydrated (Knox et al., 1975). The S_2-RNase is apparently not degraded by these proteases because SDS-PAGE revealed no significant degradation after 3 hr of incubation with pollen (Figure 3). Neither is the *S*-RNase degraded by intracellular proteases because material extracted from pollen tubes after incubation with *S*-RNase had the same molecular mass (32 kD) as the isolated *S*-RNase (Figures 4 and 6). Because there was no indication that S_2-RNase is degraded by pollen proteases, the radioactive material inside the pollen tubes shown in Figure 1 is likely to represent essentially intact S_2-RNase. To quantify the amount of S_2-RNase inside pollen tubes (in the cytoplasm and walls), pollen tubes were either treated with proteinase K or rinsed in a medium containing 100 mM NaCl to reduce the amount of *S*-RNase on their outer surface. Protease treatment has also been used to demonstrate the uptake of radioactive proteins into chloroplasts (Mishkind et al., 1987). The salt rinse was designed to reduce nonspecific charge interactions between the basic S_2-RNase and negatively charged components of the pollen tube wall, such as pectins. Rinsing pollen tubes in this medium was the most effective treatment for removing *S*-RNase from the outer surface of

Table 2. Effect of Added RNase on Translation of Pollen Tube (Genotype S_2) RNA in a Wheat Germ Cell-Free Extract

RNase Added to Incubation Mixture (Units/mL)	Incorporation of ^{35}S-Methionine into Protein (% of Incorporation Obtained in the Absence of Added RNase).		
	S_2-RNase	S_6-RNase	RNase A
10.0	1.3	1.1	0.9
1.0	2.8	2.3	8.3
0.1	13.8	24.8	33.9
0.01	67.6	73.7	77.4
0.0	100.0	100.0	100.0

These results are typical of those obtained in three separate experiments using different preparations of the RNases.

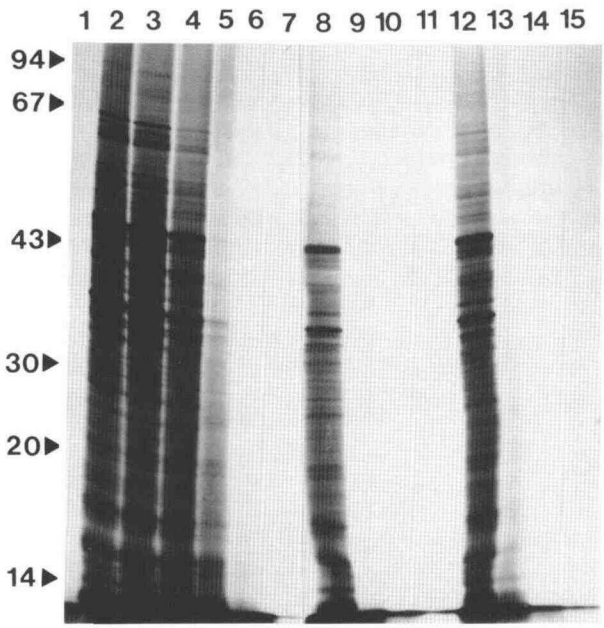

Figure 7. In Vitro Translation Products of S_2 Pollen Tube RNA after Incubation in the Presence of S_2-RNase, S_6-RNase, and RNase A.

The incubation mixture in lane 1 had no RNA added, lanes 2 through 15 had 2.5 μg of pollen tube RNA added. Lane 2 had no protein added. Lane 3 had 85 mg/mL BSA added. Lanes 4, 5, 6, and 7 had 0.01 unit, 0.1 unit, 1 unit, and 10 units/mL RNase A added, respectively. Lanes 8, 9, 10, and 11 had 0.01 unit, 0.1 unit, 1 unit, and 10 units/mL S_2-RNase added, respectively. Lanes 12, 13, 14, and 15 had 0.01 unit, 0.1 unit, 1 unit, and 10 units/mL S_6-RNase added, respectively. Concentration of protein added to each of the incubations in lanes 4 to 15 was made up to 85 mg/mL by the addition of BSA. Samples contained 2 μL of 10-μL incubations. Positions of molecular weight markers are shown as $M_r \times 10^{-3}$.

pollen tubes. In this way, the amount of S_2-RNase was reduced from approximately 35 μg/mg to 18 μg/mg S_2-pollen. An estimate of the proportion of this *S*-RNase in the walls and in the cytoplasm of the pollen tubes was obtained by disrupting the washed pollen tubes in Tris-HCl buffer. Approximately 50% of the S_2-RNase associated with S_2-pollen after rinsing in 100 mM NaCl medium (approximately 9 μg/mg of pollen) was present in the soluble fraction. These results are consistent with the microscopic observations that *S*-RNase is distributed in approximately equal proportions between the cytoplasm and inner wall.

Action of Heat-Treated *S*-RNase on in Vitro Growth of Pollen Tubes

The experiments reported also give some insight into the unexpected observation that heat-treated S_2-RNase has an enhanced ability to inhibit in vitro pollen tube growth (Jahnen et al., 1989a). The micrographs of Figure 5 show an increased amount of heat-treated S_2-RNase associated with the outer surface of inhibited pollen grains but no indication of increased uptake into the walls or cytoplasm compared with untreated S_2-RNase. After rinsing in 100 mM NaCl medium, approximately 4.5 times more heat-treated S_2-RNase remained associated with S_2-pollen than untreated S_2-RNase. Less than 1% of this heat-treated S_2-RNase was solubilized by disruption of pollen tubes in Tris-HCl buffer. This observation and direct microscopic observation show that most of the S_2-RNase was strongly bound to the outside of the pollen grains. This massive accumulation of *S*-RNase on the outer surface of the pollen grain may have some physical effect on the germination and growth of pollen. Certainly, the heat-treated S_2-RNase has little RNase activity so it is unlikely that its effect is mediated by enzymic activity in the cytoplasm of the pollen.

S-RNases Inhibit Translation of Pollen RNA in Vitro and in Vivo

The inhibition of in vitro translation of pollen RNA by isolated *S*-RNases (Table 2) is predictable and indicates that the *S*-RNases either degrade components of the translation system or the added pollen RNA or both. This experiment also indicates that it is unlikely that specific mRNAs are preferentially degraded by the *S*-RNases because no differences in the range or relative concentrations of the proteins synthesized in the presence or absence of

Table 3. Effect of S_2-RNase, S_6-RNase, or RNase A on Incorporation of ^{35}S-Methionine into in Vitro-Grown Pollen Tubes (Genotype S_2)

Treatment	RNase Activity of Pollen Tube Growth Media (Units/mL)	Relative Incorporation of ^{35}S-Methionine into Tube Proteins (% of Control)
Control (no RNase)	0	100.0
S_2-RNase (2 mg/mL)	166	31.7
S_6-RNase (2 mg/mL)	1076	71.0
RNase A (0.1 mg/mL)	347	90.4
RNase A (0.3 mg/mL)	1041	85.2
RNase A (0.5 mg/mL)	1736	70.0

These experiments were performed on three separate occasions with different *S*-RNase preparations, and the results shown are typical of those obtained. The average length of pollen tubes after incubation with S_2-RNase was estimated to be 50% of that of controls. After treatment with either S_6-RNase or RNase A at either 0.3 mg or 0.5 mg/mL, the length of the tubes was diminished to about 75% of that of controls. RNase A at 0.1 mg/mL had no observable effect on the length of pollen tubes.

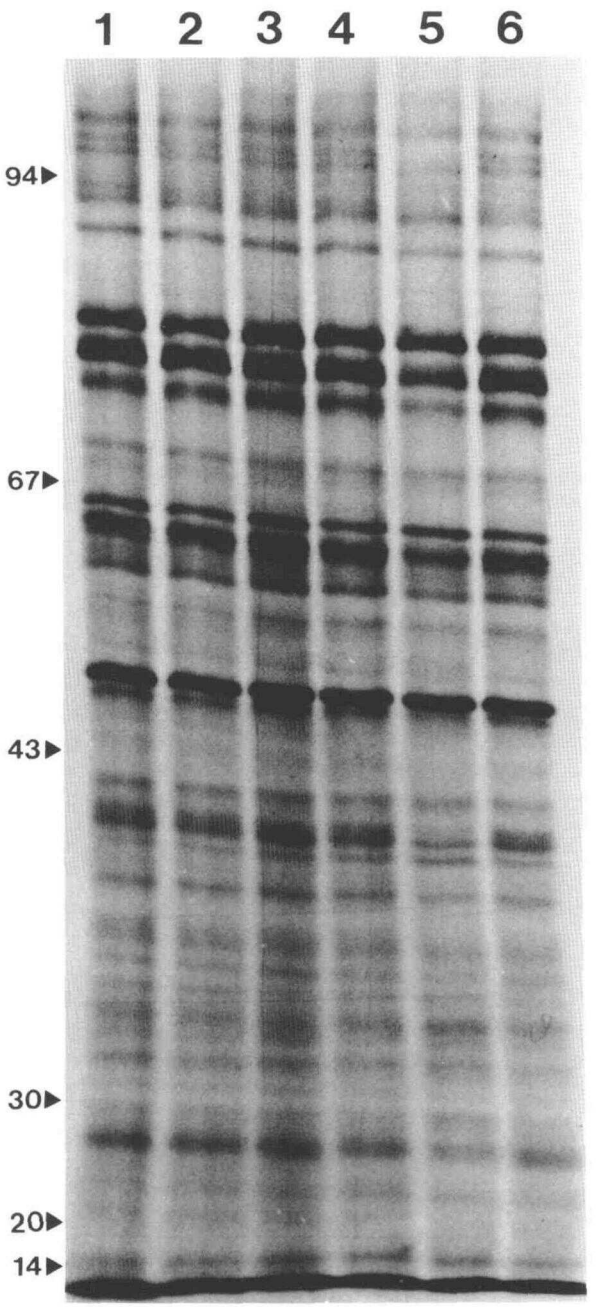

Figure 8. Incorporation of ^{35}S-Methionine into Proteins of S_2-Pollen Tubes Grown in Vitro in the Presence of S_2-RNase, S_6-RNase, and RNase A.

Lane 1 contains proteins extracted from pollen tubes incubated in the absence of exogenous RNase. Lanes 2, 3, and 4 contain proteins extracted from pollen tubes incubated in the presence of 0.1 mg, 0.3 mg, and 0.5 mg/mL RNase A, respectively. Lanes 5 and 6 contain proteins extracted from pollen tubes incubated in the presence of 2 mg/mL S_2-RNase or S_6-RNase, respectively. An equal amount of radioactivity was loaded in each lane. Positions of molecular weight markers are shown as $M_r \times 10^{-3}$.

S-RNase were detected (Figure 7). This conclusion is reinforced by the observation that the pattern of proteins synthesized by intact pollen tubes is unchanged by contact with the *S*-RNases (Figure 8). If the *S*-RNases specifically degraded a particular pollen mRNA, this would have been detected as depleted protein band(s) in the experiments shown in Figures 7 and 8. However, the finding that total protein biosynthesis within the pollen tubes, as measured by ^{35}S-methionine incorporation, is depressed (Table 3) implies that the *S*-RNase enters the tube and acts on the protein biosynthetic machinery.

Models for Interaction between the *S*-RNases and Pollen Tubes

The finding that the pistil products of the *S*-locus in solanaceous species are ribonucleases suggests that they might act as *S*-allele-specific cytotoxins (McClure et al., 1989). Consistent with this model, we recently demonstrated that pollen rRNA is degraded after incompatible pollination (McClure et al., 1990). However, these in vivo studies do not unambiguously show that *S*-RNases are responsible for this effect. The in vitro experiments reported here have an important bearing on developing a model for the mechanism of self-incompatibility. One possible model is based on the premise that *S*-RNases have a stringent substrate specificity in vivo, analogous to the DNA restriction endonucleases. In this model, the *S*-RNases might each be directed toward a particular mRNA, perhaps an *S*-allele-specific pollen RNA, the hydrolysis of which leads to inhibition of incompatible pollen tube growth. Our results do not support this model because *S*-RNase appears to affect all translation products equally, regardless of whether a cell-free system (Figure 7) or intact pollen tubes (Figure 8) are examined.

However, the data are consistent with a model involving a specific uptake mechanism. In this model, the pollen product of a particular *S*-allele might act as a specific receptor for the self *S*-RNase and would facilitate its entry to the pollen tube cytoplasm. The data indicate that the *S*-RNase is not substantially degraded during uptake into the cytoplasm or during the initial period within the cytoplasm.

In considering the experimental evidence, it is important to bear in mind the differences between the in vivo self-incompatibility system and the in vitro pollen tube growth assay. In vivo, the *S*-allele specificity of the self-incompatibility reaction is complete, in that incompatible pollen tubes rarely penetrate more than 1 cm into the style, whereas compatible tubes typically grow more than 5 cm to effect fertilization. Tracer experiments in which ^{32}P-labeled pollen was used in in vivo pollinations also show clear specificity, in that rRNA of pollen is degraded in incompatible but not compatible pollinations (McClure et al., 1990). The lack of specificity observed in vitro, such

as the finding that pollen tubes of all three *S*-genotypes take up S_2-RNase in vitro (Figures 1 and 2), and the inhibition of protein biosynthesis in S_2-pollen tubes by both S_2-RNases and S_6-RNases (Figure 8 and Table 3) do not refute the model for allele-specific uptake of *S*-RNases in vivo. Jahnen et al. (1989a) demonstrated that in vitro there is only partial expression of allelic specificity because the growth of pollen tubes of a particular genotype was inhibited to a greater extent by *S*-RNase of the same genotype than by that of a different genotype. However, growth of tubes of each *S*-genotype was inhibited, to a certain extent, by the presence of each *S*-RNase. The present data are consistent with this partial specificity. The disturbed growth and wall composition (Yi-Qin and Linskens, 1983; Vasil, 1987) of in vitro-grown tubes compared with in vivo-grown tubes could lead to perturbation of any specific cell surface receptors. In this context, the observation that in vitro-grown pollen tubes take up a diverse range of macromolecules is relevant (Hess et al., 1974).

In summary, the experiments show that *S*-RNases may enter the cytoplasm of in vitro-grown pollen tubes, that the *S*-RNases are not degraded to any observable extent, and that protein biosynthesis within pollen tubes is inhibited. The experiments also indicate that specificity is unlikely to involve a specific pollen mRNA substrate. The key question of how specificity in the interaction is controlled is not answered by this set of experiments; indeed, the experiments raise a series of questions relating to the nature of uptake of exogenous materials by plant cells, transport across walls, and the relationship between cell walls, membranes, and biosynthesis of wall components. Knowledge of these processes, as well as an understanding of the nature of the pollen product of the *S*-gene, will be necessary for our ultimate understanding of the mechanisms underlying self-incompatibility.

METHODS

Plant Material

Self-incompatibility genotypes S_2S_2, S_3S_3, and S_6S_6 of *Nicotiana alata* Link et Otto (sources as in Anderson et al., 1989) were grown under glasshouse conditions as previously described (Anderson et al., 1986). Pollen was collected soon after the flowers opened and was used fresh. For each experiment, pollen was pooled from three flowers of the same genotype. Styles of flowers were frozen and stored at −70°C.

In Vivo Radiolabeling of S_2-RNase

To obtain radiolabeled S_2-RNase in an enzymically active form, pistil proteins were labeled in vivo by applying tritiated amino acids to the stigma surface. S_2S_2 flowers were picked 1 day to 3 days before anthesis, placed in an unsealed, moist container at room temperature, and left undisturbed for 1 hr before application of ^{3}H-amino-acid mixture (1 mCi/mL overall activity, 200 μCi/mL each of leucine, lysine, phenylalanine, proline, and tyrosine; Amersham, code TRK.550) directly to the stigma surface (1 μL/stigma). After 6 hr at room temperature, a second aliquot of ^{3}H-amino acids was applied. After 24 hr, the upper 2 cm of the pistils were removed and frozen at −70°C. S_2-RNase was extracted and purified by ammonium sulfate fractionation and cation exchange chromatography, as described by Jahnen et al. (1989b). Protein concentrations were estimated by the method of Bradford (1976) using BSA as a standard. RNase activity was assayed by perchloric acid solubilization (McClure et al., 1989), and radioactivity was determined by liquid scintillation counting in an LKB 1211 Minibeta counter.

Five hundred flowers were labeled and S_2-RNase was isolated from the top 2 cm of the pistil (5.2 g of tissue) (yield, 5 mg of S_2-RNase; 5.2×10^5 cpm/mg). RNase activity was approximately 170 units/mg, which is comparable with that of a sample of unlabeled S_2-RNase assayed at the same time.

Alternative methods of labeling by chemical methylation using $NaCNBH_3$ and ^{14}C-formaldehyde (Jentoft and Dearborn, 1980) resulted in considerable loss of RNase activity and were not used.

Isolation of *S*-RNases

S_2-Glycoproteins and S_6-glycoproteins were purified from styles of *N. alata* by ammonium sulfate fractionation and cation exchange chromatography (Jahnen et al., 1989b) and stored at −70°C. Protein concentrations were determined by the method of Bradford (1976), using a Bio-Rad protein concentration determination kit and BSA as a standard. RNase activities of *S*-RNases and RNase A were determined by a perchloric acid solubilization assay (McClure et al., 1989).

In Vitro Growth of Pollen Tubes

Pollen was suspended by vortexing in growth medium made double strength in all components except MES, which was single strength [1 × growth medium = 20 mM Mes-KOH, pH 6.0, 0.07% $Ca(NO_3)_2 \cdot 4H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% KNO_3, 0.01% H_3BO_3, 2% sucrose, and 15% PEG 4000 (Jahnen et al., 1989a)] in a 1.5-mL Eppendorf tube. Fifty microliters of this pollen suspension was added to either 50 μL of 20 mM Mes-KOH, pH 6.0 (as a control incubation), 50 μL of *S*-RNase, 20 mM Mes-KOH, pH 6.0, or 50 μL of bovine pancreatic RNase A (Sigma), 20 mM Mes-KOH, pH 6.0. Contents were mixed by vortexing and incubated in the dark at 25°C for 3 hr in 1.5-mL Eppendorf tubes. Aliquots were removed to check pollen tube growth using a light microscope at ×10 or ×40 magnification. Pollen tubes were collected by centrifugation at 13,000*g* in a microcentrifuge.

Preparation of Samples for Light Microscopy

Pollen was collected from plants of SI genotypes S_2S_2, S_3S_3, and S_6S_6, and grown at a concentration of approximately 2 mg/mL in 100 μL of growth medium containing 1.35 mg/mL of either tritiated or unlabeled S_2-RNase. Control incubations contained unlabeled S_2-RNase or no added protein. After 3 hr, S_2-pollen tubes and S_3-pollen tubes grown in the presence of *S*-RNase were about half the length of control pollen tubes (approximately 100 μm to 200

μm versus 200 μm to 300 μm). S_6-pollen tubes were less inhibited by S_2-RNase (approximately 150 μm to 250 μm). There was no discernible difference in the length of pollen tubes grown in the presence of radiolabeled or unlabeled S_2-RNase. Pollen tubes were rinsed twice in fresh growth medium and fixed in 2% formaldehyde (Ladd, Burlington, VT), 2.5% glutaraldehyde (Serva, Heidelberg, Germany), 0.06 M Pipes (Sigma), pH 7.2, for 14 hr. Pollen tubes were pelleted by centrifugation and rinsed twice in 0.06 M Pipes, pH 7.2, post-fixed in 1% OsO4, 0.06 M Pipes, pH 7.2, for 1 hr and rinsed repeatedly in Pipes buffer. After a final rinse in H_2O, pollen tubes were gently resuspended in 10 mL of H_2O and embedded in small blocks of 1% agar. Samples were dehydrated by transferring agar blocks along an ethanol/H_2O series from 10% to 100% ethanol infiltrating with Spurrs resin (Spurr, 1969) mixed with ethanol in three increasing steps, and finally polymerized in 100% Spurrs resin at 70°C for 24 hr. Sections 1.5 μm thick were placed on glass slides and coated with a thin layer of liquid photographic emulsion (Kodak). Slides were developed after 30 days of exposure and examined with an Olympus BH2 microscope.

Preparation of Samples for Electron Microscopy

Because radiographic exposure times for electron microscopy sections would have been long (estimated 10 months or longer), we used immunogold cytochemical methods to localize S-RNase taken up by pollen. Pollen was grown at a concentration of approximately 4 mg/mL in 100 μL of growth medium containing 2 mg/mL S_2-RNase. Rinsed pollen tubes were fixed in 2% formaldehyde, 0.5% glutaraldehyde, 0.06 M Pipes, pH 7.1, for 2 hr. Dehydration was carried out using an ethanol/H_2O series 10% to 90%. Samples were embedded in LR gold (London Resins gold) by infiltrating with three increasing concentrations of resin in ethanol and then transferring into 2.5-cm diameter glass tubes and infiltrating with pure LR gold for 2 days with constant rotation. Fresh LR gold and 0.1% Benzil (an α-diketone, light-sensitive initiator) were mixed for final embedding of pollen tubes between Teflon-coated microscope slides and coverslips. The resin was polymerized 10 cm from a UV lamp (Philips 15W G15,T8) at −30°C. The thin layers of resin containing the flat embedded pollen tubes were remounted on polymerized LR gold blocks. Sections 80 nm to 90 nm thick were collected on pioloform coated gold grids. Sections were blocked in phosphate-buffered saline (PBS, 0.5 M NaCl), 1% BSA (Sigma A.4503), and incubated with 1.5 mg/mL anti-S_2-peptide antibody, raised in sheep (previously described by Anderson et al., 1989), in PBS, 0.1% BSA for 1 hr at 20°C. Sections were rinsed and labeled with anti-sheep IgG conjugated to 15 nm diameter colloidal gold (EY Labs, San Mateo, CA) at a 1:20 dilution in PBS, 0.1% BSA for an additional hour. For the control, S_2-antibody was preincubated with the synthetic S_2-peptide at 0.35 mg/mL for 30 min. Sections were rinsed in PBS and then in H_2O, counterstained with aqueous uranyl acetate and lead citrate, and examined in a Joel 1200 electron microscope.

Extraction and SDS-PAGE of Proteins

Pollen (approximately 3.7 mg/mL) was grown in 1.35 mg/mL [^{3}H] S_2-RNase. Rinsed pellets of pollen tubes were frozen in liquid N_2 and homogenized by grinding with three cycles of freeze thawing. SDS-PAGE loading buffer (25% glycerol, 5% 2-mercaptoethanol, 3% SDS, 62.5 mM Tris-HCl, pH 6.8) was added, samples were boiled for 10 min and vortexed, and insoluble debris was removed by centrifugation at 13,000*g*. Several procedures were used to rinse pollen tubes before extraction for experiments shown in Figures 3, 4, and 6, as follows: (1) Pollen tubes were rinsed four times in fresh growth medium, and pelleted in a microcentrifuge at 13,000*g*, supernatant was removed, and pollen tubes were gently resuspended at each step, or (2) pollen tubes were rinsed once in growth medium, then incubated 15 min on ice in 0.2 mg/mL proteinase K (Boehringer) in growth medium. Protease was inhibited by the addition of phenylmethylsulfonyl fluoride to 0.25 mM, and pollen tubes were rinsed again in 0.25 mM phenylmethylsulfonyl fluoride in growth medium. Proteinase K was able to degrade S_2-RNase at 0.5 mg/mL under these conditions, whereas trypsin and *Staphylococcus aureus* V8 protease were not (results not shown). (3) Alternatively, pollen tubes were rinsed once in growth medium, then rinsed twice in growth medium that contained no sucrose but contained 100 mM NaCl. Sucrose was omitted to maintain the osmotic potential within a range that did not result in plasmolysis of the pollen tubes. Denatured protein samples were separated by SDS-PAGE using the buffer system of Laemmli (1970). Gels were treated with Amplify (Amersham) as directed and dried, and fluorography was carried out at −70°C.

Heat Treatment of S-RNase

S_2-RNase (1.35 mg/mL) was placed in a boiling water bath for 15 min and cooled on ice for 5 min before use.

Cell-Free Translation of Pollen Tube RNA

Pollen (genotype S_2) was germinated and grown in vitro for 3 hr, pelleted in a microcentrifuge, frozen in liquid nitrogen, and homogenized in 200 μL of 0.2 M Tris-HCl, pH 8, 0.2 M sucrose, 60 mM NaCl, 30 mM $MgCl_2$, 1% SDS. The solution was extracted twice with a phenol:chloroform 1:1 mix. Nucleic acids were precipitated from ethanol as the sodium salt, precipitated twice from 4 M LiCl, and precipitated again from ethanol as the sodium salt. The RNA pellet was rinsed in ethanol, dried, and resuspended in H_2O. Total RNA (2.5 μg) was incubated in a wheat germ cell-free translation system (Promega) in the presence of 5 μCi of L ^{35}S-methionine (Amersham), in a volume of 10 μL for 1 hr at 25°C as directed by the manufacturer, except that RNase inhibitor was omitted, and 0.0001 unit to 0.1 unit of RNase A, S_2-RNase, or S_6-RNase was added per 10-μL incubation. (The final amount of protein added to each incubation was made up to 0.85 μg with acetylated BSA.) Control incubations contained 0.85 μg of BSA and no RNase; no RNase or BSA; and no RNase, BSA, or RNA. Specific activities of RNases used in these experiments were 6598 units/mg for RNase A, 118 units/mg for S_2-RNase, and 885 units/mg for S_6-RNase.

Incubations were diluted with SDS-PAGE loading buffer (25% glycerol, 5% 2-mercaptoethanol, 3% SDS, 62.5 mM Tris-HCl, pH 6.8) and boiled for 10 min, and insoluble material was pelleted in a microcentrifuge. An equal volume of each supernatant (corresponding to 2 μL of original incubation) was separated on a 12.5% SDS polyacrylamide gel. Proteins were fixed in 40% methanol-

10% acetic acid, gels were dried, and autoradiography was carried out at −70°C. To determine the amount of radioactivity incorporated, aliquots of samples were spotted onto filter paper, and unincorporated amino acids were removed by batch washing in 10% TCA on ice, 5% boiling TCA, 5% TCA on ice, and ethanol. Radioactivity was determined by liquid scintillation counting.

Labeling and Extraction of Pollen Tube Proteins

S_2-Pollen (0.25 mg/mL) was incubated in growth medium (100 μL) in the presence of S_2-RNase (83 units/mg), S_6-RNase (538 units/mg), and RNase A (3472 units/mg). After 2.5 hr of incubation, samples (1 μL) were removed to estimate pollen tube growth. ^{35}S-methionine (1 μL; 10 mCi) was added and incubation continued for an additional 30 min. Pollen tubes were rinsed in fresh growth medium, frozen in liquid nitrogen, homogenized in 4% SDS, 5% 2-mercaptoethanol, 5% sucrose, 10 mg/mL Polyclar AT, boiled for 2 min, and vortexed, insoluble matter was pelleted in a microcentrifuge, and proteins were precipitated from the supernatant in 80% acetone at −20°C. The protein pellet was dried and resuspended in SDS-PAGE loading buffer. After boiling and centrifuging samples, incorporated radioactivity of aliquots was determined. Protein samples containing equal radioactivity (200,000 cpm/track) were separated on a 10% SDS-polyacrylamide gel, and autoradiography was carried out as above.

ACKNOWLEDGMENTS

J.E.G. was supported by a fellowship from The Royal Society. We are grateful to all of our colleagues at Plant Cell Biology Research Centre, especially Dr. Geoffrey McFadden for his advice on microscopy, Bruce McGinness and Susan Mau for growing the plants, and Drs. Stephen Read, Volker Haring, and Chen Chao-Guang for critical evaluation of the manuscript.

Received November 1, 1990; accepted December 31, 1990.

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