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Expression of a Self-Incompatibility Glycoprotein (S_2-Ribonuclease) from *Nicotiana alata* in Transgenic *Nicotiana tabacum*

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In *Nicotiana alata*, self-incompatibility is controlled by a single locus, designated the *S*-locus, with multiple alleles. Stylar products of these alleles are ribonucleases that are secreted mainly in the transmitting tract tissues. *N. tabacum* plants were transformed with constructs containing the S_2-cDNA and genomic S_2-sequences from *N. alata* that were linked to the cauliflower mosaic virus 35S promoter. Unlike other genes controlled by this promoter, the genes were expressed most highly in mature floral organs. This pattern of expression was observed at both the protein and RNA levels. The S_2-glycoprotein was detected in the stylar transmitting tract tissues of the transgenic plants. The transgene product was secreted, had ribonuclease activity, and was glycosylated with the correct number of glycan chains. However, the maximum level of S_2-glycoprotein in styles of the transgenic plants was approximately 100-fold lower than that found in *N. alata* styles carrying the S_2-allele. Perhaps because of this lower protein level, the plants showed no changes in the incompatibility phenotype.

INTRODUCTION

Many flowering plants possess genetically controlled self-incompatibility (SI) systems that prevent inbreeding (for reviews, see de Nettancourt, 1977; Dickinson, 1990; Haring et al., 1990). SI is often controlled by a single, multiallelic "S"-locus. In gametophytically determined systems such as those found in the Solanaceae, pollen expresses its own haploid genotype and matings are incompatible if the *S*-allele of the pollen is matched by one of the two *S*-alleles in the diploid tissue of the style.

cDNAs corresponding to three *S*-alleles of *Nicotiana alata* have been cloned and sequenced (Anderson et al., 1986, 1989). Genomic clones of the S_2- and S_6-alleles have also been isolated and sequenced (S.-L. Mau, unpublished data). The genes encode allele-specific ribonucleases (McClure et al., 1989) that are expressed in the secretory tissues of mature pistils (Cornish et al., 1987).

Ribonuclease activity is conserved in the products of stylar *S*-alleles from *Petunia inflata* (Ai et al., 1990) and *Solanum chacoense* (Xu et al., 1990) as well as *N. alata*. It has been proposed that these ribonucleases act as *S*-allele–specific cytotoxins targeted against the protein biosynthetic apparatus of self-pollen tubes (McClure et al., 1990; Gray et al., 1991). The nature of the pollen component of the SI system is not yet understood.

This paper describes the transformation of the self-compatible species *N. tabacum* with constructs containing the S_2-cDNA and genomic S_2-sequences from *N. alata*, linked to the cauliflower mosaic virus 35S promoter.

RESULTS

Construction of Chimeric Genes and Transformation of *N. tabacum*

Three chimeric genes were constructed. Two contained the unmodified 35S promoter ligated directly upstream of a full-length S_2-cDNA (Anderson et al., 1986) in both sense and antisense orientations. The 775-bp S_2-cDNA sequence, flanked by EcoRI linkers (Anderson et al., 1986), was inserted by blunt end ligation into a unique HincII site downstream of the 35S promoter in pIC35/A. The 2.3-kb chimeric genes, containing the 35S promoter, the S_2-cDNA (sense and antisense), and a T-DNA polyadenylation sequence, were isolated on BglII fragments and inserted into a unique BglII site within the T-DNA region of the binary vector pH575 to produce the 35S–S_2-

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cDNA constructs shown in Figure 1A. The third chimeric gene contained the 35S promoter with a duplicated enhancer (Kay et al., 1987) that was ligated upstream of a genomic DNA fragment containing the coding region and 3′ end of the S_2-gene (the 35S–genomic S_2 construct, Figure 1B). The 35S–genomic S_2 construct included a single intron of 94 bp within the coding region and 0.79 kb of noncoding sequence from the 3′ end of the gene. The 5′ end of the clone was deleted to a position 90 bp downstream of the ATG, and the 5′ end of the S_2-cDNA was inserted so that an intact copy of the S_2-coding region and 3′ end was produced. This gene was inserted downstream of the modified 35S promoter in pGA55A-205 to produce the 35S-genomic S_2 construct (Figure 1B).

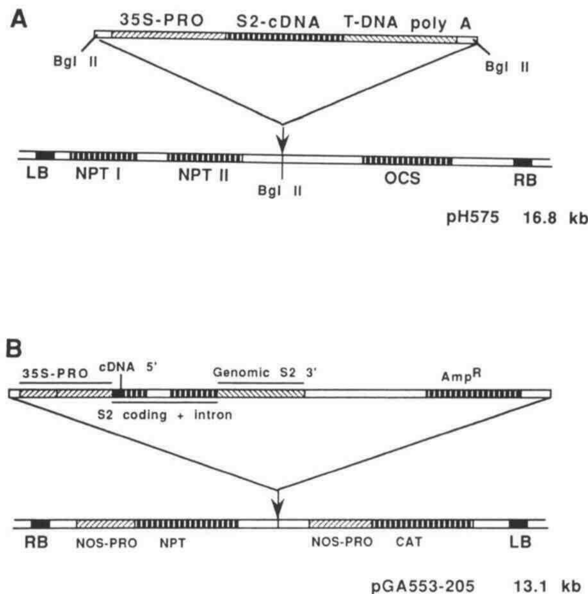

Figure 1. Construction of Binary Vectors Containing the 35S-S_2-Gene Constructs.

(A) Diagram showing the T-DNA region of the binary vector pH575, with a 35S-S_2-cDNA chimeric gene inserted into the unique BglII site. The 35S-S_2-cDNA genes contain the S_2-cDNA sequence inserted between the 35S promoter (35S-PRO) and a T-DNA–derived polyadenylation sequence (poly A), in both sense and antisense orientations. The T-DNA region of pH575 contains an octopine synthase gene (OCS) and two neomycin phosphotransferase (NPT) genes that conferred resistance to kanamycin in both plants (NPT II) and bacteria (NPT I).
(B) Diagram showing the T-DNA region of the binary vector pGA553-205 (An et al., 1986), with the chimeric 35S–genomic S_2-gene inserted into the polylinker site. The 35S–genomic S_2-gene contains the 35S promoter with the −90 to −440 region duplicated. This was linked to the 5′ end of the S_2-cDNA. A unique HpaI site located 90 bp downstream of the ATG was used to ligate the genomic S_2-clone (S.-L. Mau, unpublished data) in such a way that an intact copy of the S_2-coding region, including the intron, was produced. The construct includes 790 bp of genomic sequence 3′ of the S_2-coding region. The chimeric gene, together with pGEM-Blue sequence (Promega, including an ampicillin resistance gene [Amp^R]), was inserted between the chloramphenicol acetyltransferase (CAT) gene and the neomycin phosphotransferase (NPT) gene in the T-DNA region of the binary vector. RB, right T-DNA border; LB, left T-DNA border; NOS-PRO, nopaline synthase promoter.

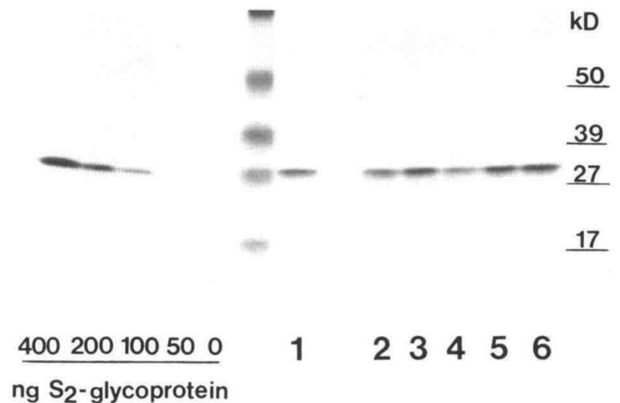

Figure 2. Protein Gel Blot Analysis of Mature Style Extracts from *N. tabacum* Plants Transformed with the 35S–Genomic S_2 Construct.

Lane 1 contains extract from mature *N. tabacum* styles (150 μg of total soluble protein) of a primary transformant expressing the 35S–genomic S_2 construct. Lanes 2 to 6 contain mature style extracts (150 μg of total soluble protein) from five progeny produced by selfing of the primary transformant. For reference, the SDS–polyacrylamide gel also contained a dilution series of purified S_2-glycoprotein mixed with style extract (50 μg of total soluble protein) from untransformed *N. tabacum*. The amounts of S_2-glycoprotein in each lane are indicated. The proteins were transferred to nitrocellulose and probed with an S_2-peptide–specific antibody as described in Methods. All plants produced similar levels of S_2-glycoprotein.

N. tabacum plants were transformed with these constructs using the Agrobacterium-mediated leaf disc transformation system as described in the Methods. Eighty-nine transgenic tobacco plants were produced: 71 containing the correct sense 35S–S_2-cDNA construct, 11 containing the antisense construct, and seven containing the 35S–genomic S_2 construct. DNA gel blot analysis of 11 plants indicated that nine were transformed with intact copies of the chimeric genes (data not shown).

All plants transformed with the correct sense chimeric genes were screened for expression of the S_2-glycoprotein in styles using protein gel blots probed with an S_2-peptide–specific antibody. Sixteen plants transformed with the 35S–S_2-cDNA construct, and one plant transformed with the 35S–genomic S_2 construct produced the S_2-glycoprotein at detectable levels. The highest level of expression was found in the plant transformed with the 35S–genomic S_2 construct. Figure 2 shows a protein gel blot of style extracts from mature flowers of this plant and five of its kanamycin-resistant self-progeny. All six plants produced similar levels of the 32-kD S_2-glycoprotein in their styles. The blot included a dilution series of purified S_2-glycoprotein isolated from *N. alata*, from which the level of expression in the transgenic plants was estimated to be between 0.1 and 0.3% of total soluble protein.

Analysis and Localization of the S_2-Glycoprotein Produced in Styles of Plants Transformed with the 35S-Genomic S_2 Construct

The S_2-glycoprotein was partially purified from 500 transformed styles by cation exchange, fast protein liquid chromatography. The S_2-glycoprotein in the partially purified sample was estimated on a protein gel blot to account for approximately 10% of the total protein (data not shown). The specific ribonuclease activity of the partially purified protein (10.3 units per mg of protein) was compared with that of purified S_2-glycoprotein from *N. alata* (161.0 units per mg of protein), as shown in Table 1. Ribonuclease activities of unfractionated style extracts from transgenic plants and untransformed *N. tabacum* plants were also compared. The mean specific activity of the transgenic plant extracts (1.94 units per mg of protein) was 1.7 times that of the extracts from untransformed *N. tabacum* (1.12 units per mg of protein).

The *N*-glycosylation pattern of the S_2-glycoprotein produced by the transgenic plants was compared with that of the S_2-glycoprotein produced by *N. alata*. A time course of hydrolysis of the two protein samples by peptide *N*-glycosidase F (*N*-glycanase) showed very similar products on a protein gel blot, as shown in Figure 3.

The S_2-glycoprotein was localized within the styles of the transgenic plants by immunocytochemistry, as shown in Figure 4. The construct was expressed in all tissues; however, most S_2-glycoprotein appeared to accumulate in the transmitting tract tissues.

Table 1. Ribonuclease Activities of Protein Samples from Styles of *N. alata* Genotype S_2S_2 and *N. tabacum* Plants Transformed with the 35S-Genomic S_2 Construct

Sample	Protein Assayed (μg)	Mean Specific Activity[a] (U/mg protein)
Partially purified S_2-glycoprotein from transformed styles	2	10.3
Purified S_2-glycoprotein from *N. alata* genotype S_2S_2 styles	0.2	161.0
Total style extract from transformed plants[b]		
A	20	1.98
B	20	1.98
C	20	1.85
Total style extract from untransformed *N. tabacum* plants[b]		
D	20	1.23
E	20	1.08
F	20	1.06

[a] See Methods for specific activity determination, which is given in units per milligram of protein.

[b] Three separate extracts from these plants were assayed in duplicate.

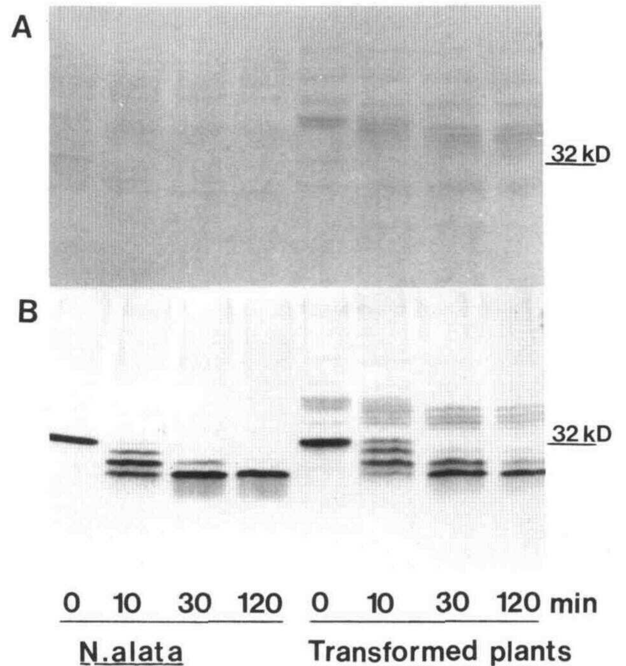

Figure 3. Treatment of the S_2-Glycoprotein from Plants Transformed with the 35S-Genomic S_2 Construct by Incubation with *N*-Glycanase.

The products formed during a time course of incubation of S_2-glycoproteins with *N*-glycanase were analyzed on a protein gel blot. Lanes marked *N. alata* contain the S_2-glycoprotein purified from *N. alata* (200 ng per lane) mixed with style extract from untransformed *N. tabacum* (3.2 μg of total soluble protein per lane) to prevent mobility artifacts caused by different loading. The lanes marked "transformed plants" contain partially purified proteins from styles of plants expressing the 35S-genomic S_2 construct (3.2 μg per lane). Samples were taken at 0, 10, 30, and 120 min. The proteins were size fractionated on an SDS-polyacrylamide gel and then transferred to a nitrocellulose filter.
(A) Photograph of the filter stained with Ponceau S.
(B) The same filter after it was destained and probed with an S_2-peptide-specific antibody, as described in Methods. The S_2-glycoproteins from both sources contained three *N*-linked glycan chains of similar sizes. Two other glycoproteins (approximately 37 and 38 kD) were more abundant than the S_2-glycoprotein in the partially purified protein mixture from the transgenic plants. These glycoproteins were also hydrolyzed by *N*-glycanase.

Styles were dissected to separate transmitting tract and stigmatic tissues from cortical, vascular, and epidermal tissues as described in the Methods. The tissues were separated under an isotonic buffer so that soluble secreted proteins collected in the buffer. Proteins were extracted from the tissue fractions, and the amount of S_2-glycoprotein present in each of the fractions was determined by protein gel blot analysis using the S_2-peptide-specific antibody as a probe, as shown in Figure 5. The S_2-glycoprotein was detected only in the secreted fraction from styles of the transgenic *N. tabacum* plants, at a level of approximately 0.2 to 0.4% of total soluble protein. Similarly,

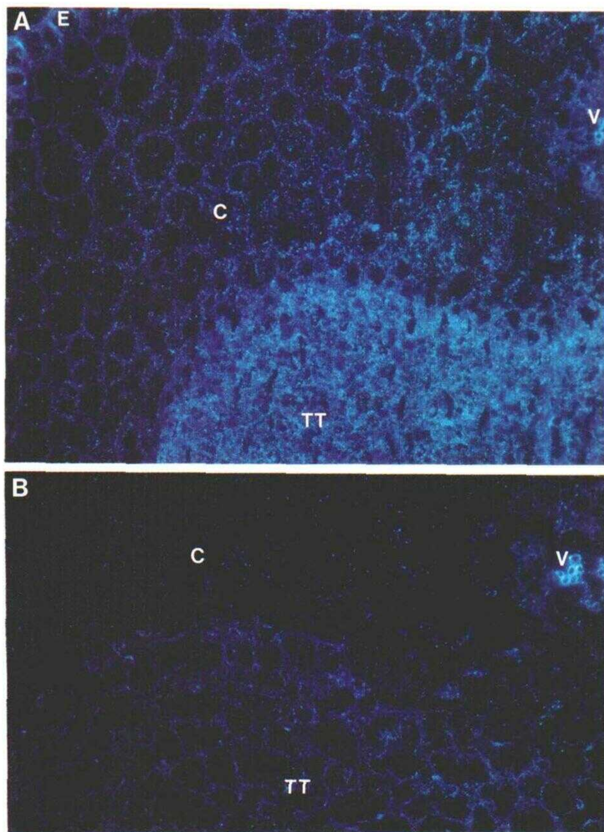

Figure 4. Localization of the S_2-Glycoprotein within the Stylar Tissues of the Transgenic Plants.

(A) *Transverse section* through the style of a plant transformed with the 35S-genomic S_2 construct.

(B) Transverse section through the style of an untransformed *N. tabacum* plant.

Transmitting tract (TT), cortical (C), epidermal (E), and vascular (V) tissues are shown. The sections were labeled with the S_2-peptide-specific antibody and immunogold markers, and photographed under polarized light. All tissues of the transgenic style were labeled, with the heaviest labeling in the transmitting tract. The untransformed style shows background labeling. Lignified cell walls in vascular tissues of both sections also reflected the polarized light.

the S_2-glycoprotein was detected primarily in the secreted fraction of *N. alata* styles (approximately 40% of total soluble protein). A trace of S_2-glycoprotein was detected in the transmitting tract fraction from *N. alata* styes when 5 μg of that protein was loaded. In the transmitting tract fraction of the transgenic styles, a band corresponding to a higher molecular mass (approximately 34 kD) than that of the S_2-glycoprotein also bound the anti-S_2 antibody. This band was visible, though less intense, in all other lanes containing style extracts, including those from untransformed *N. tabacum*.

Comparison of S_2-Gene Expression Levels in Transgenic Plants with Those of *N. alata*

The levels of S_2-glycoprotein in styles at different developmental stages from *N. alata* genotype S_2S_2 were analyzed on a

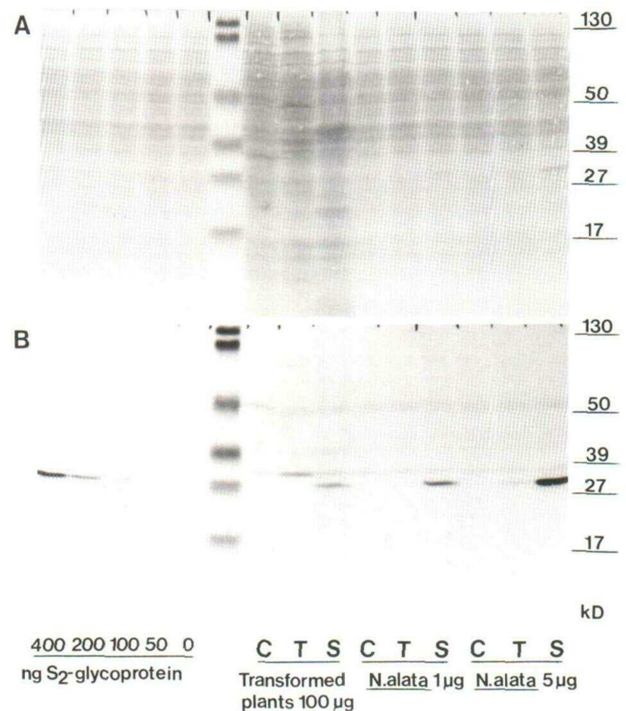

Figure 5. Protein Gel Blot Analysis of Tissue Fractions from Styles of *N. alata* Genotype S_2S_2 and Plants Transformed with the 35S-Genomic S_2 Construct.

Lanes labeled "transformed plants 100 μg" contain extracts (100 μg of total soluble protein) from different fractions of styles from plants expressing the 35S-genomic S_2 construct. Lanes marked "*N. alata* 1 μg" and "*N. alata* 5 μg" contain extracts (1 and 5 μg, respectively, of total soluble protein) from different fractions of *N. alata* genotype S_2S_2 mixed with style extract (50 μg of total soluble protein) from untransformed *N. tabacum*. The fractions analyzed were C, including cortical, epidermal, and vascular tissues; T, including transmitting tract and stigmatic tissues; S, including soluble secreted proteins. The SDS-polyacrylamide gel also included a dilution series of purified S_2-glycoprotein mixed with style extract from untransformed *N. tabacum* (50 μg of total soluble protein). The amount of S_2-glycoprotein loaded in each lane is indicated. After electrophoresis, the proteins were transferred to a nitrocellulose filter.

(A) Photograph of the filter stained with Ponceau S.

(B) The same filter after it was destained and probed with an S_2-peptide-specific antibody. The 32-kD S_2-glycoprotein was detected primarily in secreted fractions. A band of approximately 34 kD was present in all lanes containing style extracts and was most abundant in the transmitting tract fraction of transformed plants. The nature of this protein is not known.

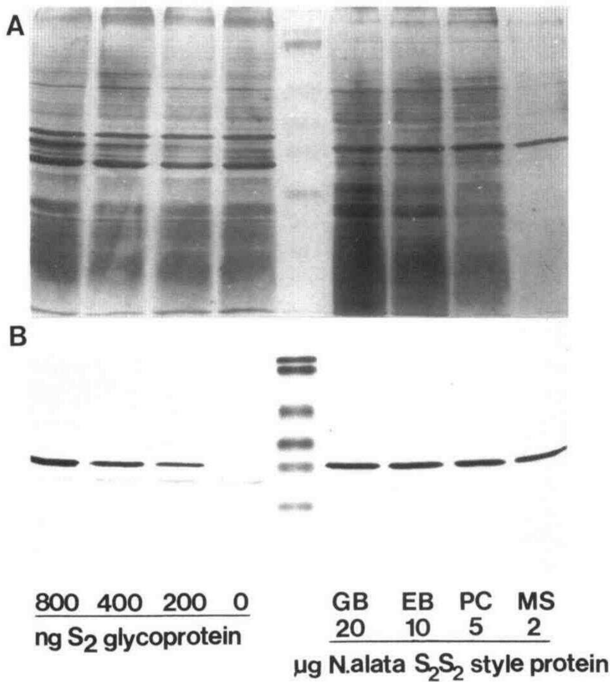

Figure 6. Protein Gel Blot Analysis of the Style Extracts from *N. alata* Genotype S_2S_2 Flowers at Different Development Stages.

Two identical SDS-polyacrylamide gels were prepared. Each included a dilution series of purified S_2-glycoprotein from *N. alata* that was mixed with style extract from *N. alata* genotype S_1S_3 (10 μg of total soluble protein). The amount of S_2-glycoprotein loaded in each lane is indicated. Extracts from styles of *N. alata* S_2S_2 flowers at the following developmental stages were loaded: green bud (GB, 20 μg of total soluble protein), elongated green bud (EB, 10 μg of total soluble protein), petal coloration stage (PC, 5 μg of total soluble protein), and mature (MS, 2 μg of total soluble protein).

(A) Photograph of one of the gels after staining with Coomassie Brilliant Blue R250.

(B) *Proteins from the other gel were transferred to nitrocellulose and* probed with an S_2-peptide-specific antibody. The level of S_2-glycoprotein in styles from green buds was approximately 2% of total soluble protein and increased with maturity.

protein gel blot, as shown in Figure 6. Bud styles contain the S_2-glycoprotein (2 to 4% of total soluble protein), and the level increases with maturity to between 20 and 40% of total soluble protein in mature styles (Figure 6).

The levels of S_2-mRNA in mature styles from *N. alata* and from the transgenic plants were compared. The level of S_2-transcript in the transformed styles was approximately fivefold less than the level found in *N. alata* S_2S_2 styles, as shown in Figure 7.

Differential Expression of the 35S Promoter Constructs in Organs of Transgenic Tobacco Plants

A protein gel blot was used to compare S_2-glycoprotein levels in leaves, corollas, styles, ovaries, and anthers, at three stages of development, from a plant transformed with the 35S-S_2-cDNA construct (Figure 8A). In general, the S_2-glycoprotein level was low in bud tissues and increased with maturity. The level was approximately 0.1% of total soluble protein in mature styles, and higher in mature corollas and anthers at anthesis. No S_2-glycoprotein was detected in mature pollen. Expression levels did not vary significantly between different leaves and were lower than in mature floral tissues (0.02 to 0.03% of total soluble protein).

An RNA gel blot was used to compare S_2-transcript levels in different organs of the transgenic plant, as shown in Figure 8B. Organs at the same stages of development as those tested for S_2-glycoprotein levels were used; however, mature pollen was not tested because not enough was collected to extract RNA. RNA from all organs of the transformed plant contained two major sequences homologous to the S_2-cDNA. One of these was slightly smaller than the major S_2-transcript found in *N. alata* styles (approximately 900 bases), whereas the other was larger (approximately 1200 bases). The pattern of expression at the RNA level was generally similar to that found at the protein level. The amount of S_2-RNA in leaves and floral buds was relatively low, and increased with maturity in floral tissues. This pattern was observed for both the large and small molecular weight transcripts. In anthers, this trend was not followed, because S_2-glycoprotein levels were higher in anthers

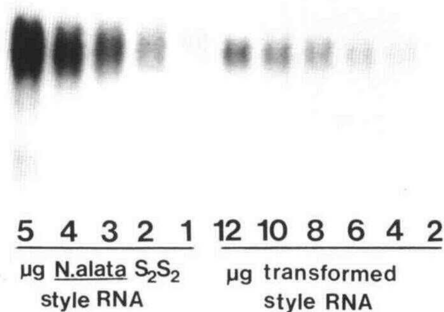

Figure 7. RNA Gel Blot Analysis of Style RNAs from *N. alata* Genotype S_2S_2 and from a Plant Transformed with the 35S-Genomic S_2 Construct.

Autoradiograph of an RNA gel blot showing dilution series of total style RNAs from *N. alata* genotype S_2S_2 and from a plant expressing the 35S-genomic S_2 construct. The amount of RNA loaded in each lane is indicated. The blot was probed with ^{32}P-labeled S_2-cDNA, as described in Methods, and exposed to x-ray film for 2 hr.

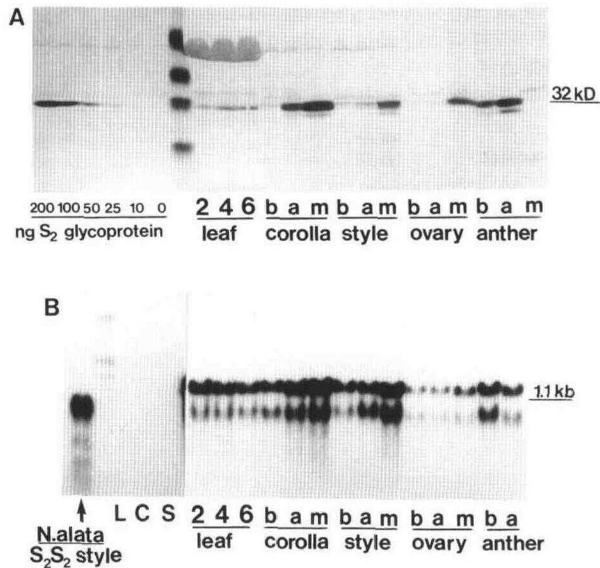

Figure 8. Protein and RNA Gel Blot Analyses of S_2-Gene Expression Patterns in Different Organs of an *N. tabacum* Plant Transformed with the 35S–S_2-cDNA Construct.

(A) Protein gel blot showing levels of S_2-glycoprotein in leaves, corollas, styles, ovaries, and anthers. Leaf numbers 2, 4, and 6 refer to positions of the leaves on the plant, with number 2 being the second leaf from the apex of a flowering stem, number 4 being the fourth leaf from the apex, and number 6 being the sixth leaf from the apex. Floral organs at three developmental stages were tested and are green bud (b), anthesis (a), and mature (m). The "mature anther" lanes contain extract from mature pollen. Each lane was loaded with 200 μg of total soluble protein extract. Control lanes contain purified S_2-glycoprotein from *N. alata* mixed with style extract (200 μg of total soluble protein) from untransformed *N. tabacum*. The proteins were size fractionated on an SDS–polyacrylamide gel, then transferred to a nitrocellulose filter, and probed with an S_2-peptide–specific antibody.
(B) Autoradiograph of an RNA gel blot showing levels of S_2-RNA transcripts in leaves, corollas, styles, ovaries, and anthers. Total RNA (11 μg) from organs at the same developmental stages as those shown in **(A)** were tested, excluding mature pollen. The lane marked "*N. alata* S_2S_2 style" contains total RNA (0.8 μg) from mature styles of *N. alata* genotype S_2S_2. Lanes marked L, C, and S contain total RNA (11 μg) from leaf 6, mature corolla, and mature style, respectively, of untransformed *N. tabacum*. The RNA was transferred to a Zetaprobe membrane filter, and the ^{32}P-labeled S_2-cDNA probe was hybridized at 68°C as described in Methods. Test lanes were exposed to x-ray film overnight, and control lanes were exposed for 6 days.

at anthesis than at bud, whereas S_2-RNA levels were higher in anthers at bud.

The pattern of expression in a second, independently transformed plant was very similar at the RNA level (data not shown). RNA from mature pollen of this plant was tested, and no S_2-transcript was detected (data not shown).

The pattern of expression of the 35S–genomic S_2 construct in leaves and floral organs of an individual transformant was also analyzed. Protein gel blot analysis, as shown in Figure 9A, showed a pattern of expression similar to that found in the plants transformed with the 35S–S_2-cDNA constructs, except that the highest level of S_2-glycoprotein was found in mature styles (0.3% of total soluble protein). Expression was low in leaves and green buds (0.02 to 0.03% of total soluble protein), and increased with maturity in floral organs. No S_2-glycoprotein was detected in mature pollen. The RNA gel blot (Figure 9B) showed a single S_2-homologous transcript similar in size to that found in *N. alata* genotype S_2S_2. The pattern of expression at the RNA level was not quite the same as in plants transformed with the 35S–S_2-cDNA construct. In general, S_2-RNA levels were higher in floral organs than in leaves, but they did not increase with maturity in all of the floral organs. The steady state S_2-RNA level in styles at anthesis was about the same as that in mature styles. S_2-RNA was more abundant in ovaries at bud than ovaries at anthesis or mature ovaries. As in the plants transformed with the 35S–S_2-cDNA construct,

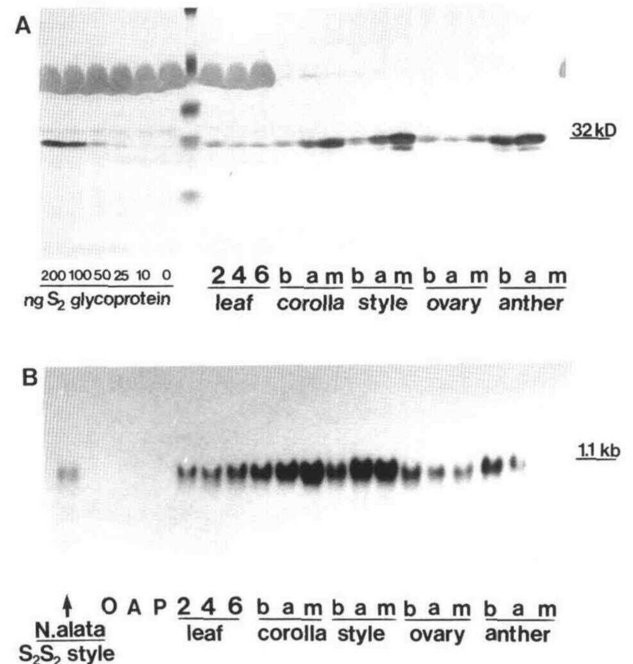

Figure 9. Protein and RNA Gel Blot Analysis of S_2-Gene Expression Patterns in Different Organs of a Plant Transformed with the 35S–Genomic S_2 Construct.

(A) A protein gel blot showing levels of S_2-glycoprotein in leaves, corollas, styles, ovaries, and anthers was analyzed as described for Figure 8A, and abbreviations are as given in Figure 8.
(B) An autoradiograph of an RNA gel blot showing levels of S_2-RNA transcripts in leaves, corollas, styles, ovaries, and anthers was analyzed as described for Figure 8B. Lanes marked O, A, and P contain total RNA (11 μg) from mature ovaries, anthers at anthesis, and mature pollen, respectively, of untransformed *N. tabacum*. The blot was probed with ^{32}P-labeled S_2-cDNA, as described in Methods, and exposed to x-ray film overnight.

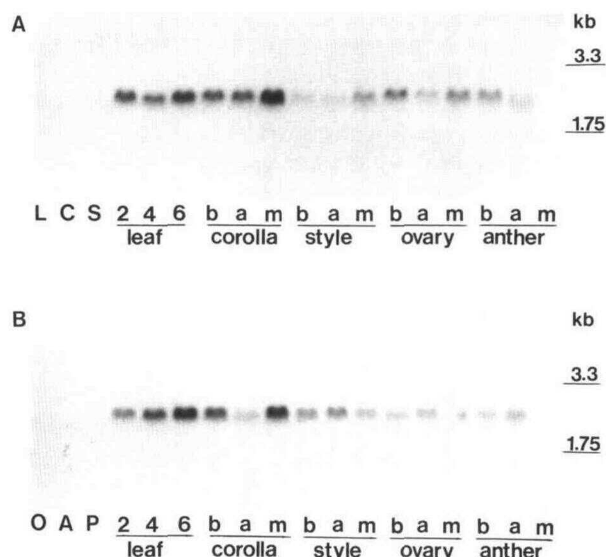

Figure 10. RNA Gel Blot Analysis of GUS Gene Expression Patterns in Different Organs of Two Plants Transformed with the 35S-GUS Construct.

Autoradiographs of RNA gel blots showing levels of GUS transcripts in leaves, corollas, styles, ovaries, and anthers of two transgenic plants were analyzed as described for Figure 8B. Each lane was loaded with 10 μg of total RNA. Lanes marked L, C, S, O, A, and P contain total RNA (10 μg) from leaves, corollas, styles, ovaries, anthers, and pollen of untransformed *N. tabacum*, respectively. The blots were probed with a ^{32}P-labeled DNA fragment containing the GUS coding region under the conditions described in Methods, and exposed to x-ray film overnight.

(A) Analysis of an *N. tabacum* plant transformed with the 35S-GUS construct.

(B) Analysis of a second, independently transformed plant.

the anthers at bud stage contained more S_2-RNA than anthers at anthesis. No S_2-transcript was detected in mature pollen.

The pattern of expression of the 35S–S_2-gene constructs was compared with that of another gene linked to the 35S promoter. Two plants transformed with a construct containing the 35S promoter linked to the β-glucuronidase gene (the 35S-GUS construct; Jefferson et al., 1987) were analyzed using RNA gel blots, as shown in Figure 10. Both plants produced a single major RNA transcript that hybridized with the GUS cDNA probe. The pattern of expression in different organs was different from the pattern observed with the S_2 constructs. Both plants showed higher levels of 35S-GUS expression in leaves and corollas compared with styles, ovaries, and anthers. In general, the level of expression in a particular organ varied little with maturity. One exception to this was in corollas at anthesis from the plant shown in Figure 10B, which showed a less intense hybridizing band than corollas at bud or maturity. However, hybridization with the 28S ribosomal RNA probe (see Methods) indicated that, unlike all other RNA samples analyzed from transgenic plants, the RNA in this lane was partially degraded (data not shown). Neither of the transgenic plants contained detectable levels of GUS RNA in mature pollen.

Pollination Behavior of the Transgenic *N. tabacum* Plants

The transgenic plants showed no change in incompatibility phenotype. The plants spontaneously self-pollinated and produced fruits containing viable seeds. They also produced fruit after pollination with *N. alata* S_2-pollen, as do untransformed *N. tabacum* plants. Both *N. tabacum* and *N. alata* pollen tubes growing within the styles of the transgenic plants were examined using fluorescence microscopy and appeared to be morphologically identical to pollen tubes growing within styles of untransformed *N. tabacum* plants (data not shown).

DISCUSSION

N. tabacum plants were transformed with constructs encoding the S_2-glycoprotein to investigate how the plants would respond to incorporation of a gene that is normally expressed only in the style and that encodes a potentially toxic product (the S_2-ribonuclease). *N. tabacum* was chosen as the host plant for this study because transformation of *N. alata* is technically difficult. Constructs driven by the cauliflower mosaic virus 35S promoter, rather than *S*-gene promoters, were used because separate studies have shown that the cloned *S*-gene promoters from *N. alata* do not function in *N. tabacum* (J. Murfett, S.-L. Mau, P.R. Ebert, V. Haring, J.-D. Faure, and A.E. Clarke, manuscript in preparation). The maximum levels of S_2-glycoprotein in the transgenic plants (approximately 0.3% of total soluble protein in styles and 10-fold lower in leaves; Figures 2 and 9) were within the range of expression levels found for other genes controlled by the 35S promoter and/or

Table 2. Expression Levels of Genes Controlled by the 35S Promoter and/or 35S Enhancer in Transgenic Tobacco

Gene	Percent of Total Soluble Protein in Leaves (Maximum Level)	Reference
Pea Vicilin	0.01	Higgins and Spencer (1991)
Human Serum Albumin	0.02	Sijmons et al. (1990)
TMV Coat Protein	0.1	Abel et al. (1986)
Cowpea Trypsin Inhibitor	1.0	Hilder et al. (1987)
Mouse Antibody	1.3	Hiatt et al. (1989)
Alfalfa Glutamine Synthase	5.0	Eckes et al. (1989)

35S enhancer, as shown in Table 2. The antisense 35S–S_2-cDNA construct was included as a control. Czako and An (1991) showed a clear difference between the regeneration efficiencies of tobacco leaf discs transformed with toxic and nontoxic gene products. By contrast, we found no differences in the regeneration efficiencies of plant material transformed with the correct sense and antisense constructs, suggesting that the correct sense construct did not inhibit shoot regeneration. This may be due to the fact that the ribonuclease is secreted.

The 35S-Genomic S_2 Gene Product Is Correctly Processed and Secreted

In *N. alata*, the S_2-RNase is secreted (Anderson et al., 1989) and glycosylated at three of the four potential sites identified by sequence analysis (Woodward et al., 1989). Our results show that the S_2-RNase produced in the transgenic plants is similarly glycosylated and deposited into the extracellular matrix within the transmitting tract of the style (Figures 3 to 5). Furthermore, the higher levels of specific ribonuclease activity in style extracts from the transgenic plants compared with those from untransformed *N. tabacum* (Table 1) indicate that the transgene encodes an active ribonuclease.

The Transgenic Plants Show No Changes in Incompatibility Phenotype

Despite the findings that the 35S–genomic S_2 gene product is correctly processed and expressed in the correct tissues, the transgenic plants show no change in incompatibility phenotype. There are two possible explanations for this. The inability of the transgenic plants to reject *N. alata* S_2 pollen may be due to the lower level of expression of the *S*-gene by the transgenic plants compared with that of the endogenous gene in *N. alata*. Alternatively, it is possible that the *N. alata* *S*-gene product cannot function in the *N. tabacum* genetic background. For example, additional modifier genes, expressed in mature styles of *N. alata* but not found in *N. tabacum*, may be necessary for *S*-associated inhibition of pollen tube growth to occur.

The maximum level of S_2-glycoprotein in styles of the transgenic plants was approximately 100-fold lower than the level in mature *N. alata* S_2S_2 styles, and 10-fold lower than the level found in styles from green buds of *N. alata* genotype S_2S_2. Immature flowers from *N. alata* do not express the self-incompatibility phenotype, and self-pollen is able to grow through the styles of these flowers. The presence of *S*-glycoproteins in styles of these flowers suggests that a threshold level may be necessary for the self-incompatibility reaction to occur. Clark et al. (1990) found evidence suggesting that threshold levels of stylar *S*-gene products are necessary for self-incompatibility in petunia, another member of the Solanaceae. They found that in *P. hybrida* genotype S_1S_2 the S_2-allele was expressed more highly than the S_1-allele during style development. These styles rejected S_2 pollen at an earlier developmental stage than they rejected S_1 pollen.

It is also possible that *N. tabacum* lacks an additional gene product(s) that is expressed in mature styles of self-incompatible plants and is needed for rejection of incompatible pollen. There are indications, both from early genetic studies and from more recent molecular studies, that additional "modifier" genes are involved in the self-incompatibility reaction. Early genetic studies showed that self-incompatibility can be lost through changes in genetic background or mutations outside the *S*-locus (for a review, see de Nettancourt, 1977). This loss of self-incompatibility may be due to loss or reduced expression of the stylar *S*-gene products or to mutations that affect pollen *S*-gene activity rather than the style component. For example, Martin (1968) showed that *S*-alleles from self-incompatible tomato species, when backcrossed into self-compatible tomato, retained their specific activity in the style but lost their activity in pollen.

Clark et al. (1990) found that a pseudo–self-compatible line of *P. hybrida* contained an *S*-glycoprotein that was expressed at similar levels to an *S*-glycoprotein from a self-incompatible line and had ribonuclease activity. Ai et al. (1991) discovered a putative *S*-allele from self-compatible cultivated *P. hybrida* that became partially active when *P. hybrida* was crossed with self-incompatible *P. inflata*. These observations may indicate that additional stylar gene products are needed for the self-incompatibility reaction. On the other hand, the inhibition of *N. alata* pollen tubes grown in vitro by isolated *S*-glycoproteins (Jahnen et al., 1989b) suggests that *S*-glycoproteins are able to act independently of other stylar gene products. Higher expression levels of the *S*-glycoproteins in transgenic *N. tabacum* plants might help to resolve this question.

The 35S-S_2-Gene Constructs Are Expressed Most Highly in Mature Floral Tissues

An unexpected finding from this study was that the expression patterns of the 35S–S_2-gene constructs in different organs of transgenic plants differed from those of other genes controlled by the 35S promoter. The plants transformed with the 35S–S_2-gene constructs showed levels of S_2-gene expression higher in floral organs than in leaves, and the expression level increased with maturity in the floral organs. This pattern was observed in three independently transformed plants, indicating that it was not due to transgene position effects. In contrast, two independently transformed plants expressing the 35S-GUS chimeric gene showed higher expression in leaves and corollas, and the expression level varied little with maturity. An et al. (1988) found that, like the GUS gene, the chloramphenicol acetyltransferase (CAT) gene was expressed more highly in leaves and corollas than other floral organs when controlled by the 35S promoter. Williamson et al. (1989) found that a 35S–zein cDNA construct was expressed more highly in immature vegetative and floral tissues than in mature tissues.

The unusual pattern of expression of the 35S–S_2-gene constructs suggests that these constructs may include sequences that contribute to the tissue-specific expression of the S_2-gene. Because the pattern is observed with the 35S–S_2-cDNA construct as well as the 35S–genomic S_2 construct, these sequences must be present within the exons of the gene. The pattern of expression is similar at the protein and RNA levels, indicating that mechanisms related to transcription and/or RNA stability, rather than protein stability, are responsible.

Evidence from many transformation experiments has indicated that regulation of gene expression is controlled mainly at the transcriptional level by *cis*-acting elements present in the 5′ regions of the genes (for a review, see Benfey and Chua, 1989); however, in some genes, sequences downstream of the transcription start site may be involved. Dean et al. (1989) showed that sequences downstream of the translation start site regulated quantitative expression of two petunia ribulose bisphosphate carboxylase genes. Nuclear run-on experiments showed that this regulation was at the transcriptional rather than post-transcriptional level. Maas et al. (1991) showed that a positive regulatory element exists in the first exon of the maize *Shrunken-1* gene. Sequences from within exons are required for the light regulation of a pea ferredoxin 1 (*Fed 1*) gene (Elliot et al., 1989) and a parsley 4-coumarate/CoA ligase (*4CL-1*) gene (Douglas et al., 1991) in transgenic tobacco. As in these examples, it is possible that the pattern of expression of the 35S–S_2-gene constructs may be at least partly due to transcriptional regulation by sequences within the exons of the S_2-gene from which the cDNA was derived.

Another important observation was the discrepancy between S_2-RNA and S_2-glycoprotein levels in styles of the transgenic *N. tabacum* plants compared with the levels found in *N. alata* S_2S_2 styles. Similar observations were made by others. For example, Ohtani et al. (1991) transformed *N. tabacum* with a chimeric gene composed of a β-phaseolin promoter and an α-zein coding sequence. α-Zein mRNA levels in the transgenic tobacco seeds at 20 days post-pollination were between 1.0 and 2.5% of the total mRNA population. However, the amount of α-zein protein varied between 1×10^{-4}% and 1×10^{-5}% of total seed protein. In vivo labeling experiments indicated that the α-zein protein was degraded in tobacco seeds with a half-life of less than 1 hr. One explanation for this low protein stability is that the β-phaseolin promoter is more active in embryonic tissues, whereas the zein protein is normally expressed in endosperm tissues (Ohtani et al., 1991).

An analogous situation could exist in the transgenic tobacco plants expressing the 35S–S_2-gene constructs. Several authors have noted that the 35S promoter is most active in vascular tissues (Benfey and Chua, 1989; Schneider et al., 1990). It is possible that within styles of the transgenic plants most of the expression of the 35S–S_2-gene constructs occurred in tissues other than the transmitting tract, where the S_2-glycoprotein may be unstable.

In the plants transformed with the 35S–S_2-cDNA construct, two S_2-transcripts of different sizes were produced (Figure 8). The S_2-cDNA clone contained a polyadenylation site at its 3′ end (Anderson et al., 1986), and a T-DNA–derived polyadenylation site was ligated downstream of this (Figure 1). It is likely that the two transcript sizes resulted from transcription termination at both of these sites. Two transcript sizes were also produced in tobacco plants transformed with a barley lectin cDNA construct that contained a T-DNA–derived polyadenylation site ligated downstream of the cDNA (Bednarek et al., 1990).

METHODS

The 35S–S_2-cDNA Constructs

The plasmid pIC35/A, containing 830 bp of 35S promoter sequence from a Cabb S strain of cauliflower mosaic virus (Odell et al., 1985), and the binary vector pH575 were kindly provided by Dr. Don Merlo, Agrigenetics Corporation, Madison, WI.

Escherichia coli MC1061 (Meissner et al., 1987) was used for vector construction and maintenance.

The 35S–Genomic S_2 Construct

The plasmid p35SPRO, containing 440 bp of 35S promoter sequence, was kindly provided by Dr. E. Dennis, Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, Australia. The binary vector pGA553-205 was kindly provided by Dr. G. An, Washington State University, Pullman.

The genomic S_2-clone from *Nicotiana alata* (S.-L. Mau, unpublished data) includes 1.6 kb of sequence 5′ of the start codon and 0.79 kb of sequence 3′ of the stop codon. A 94-bp intron interrupts the coding region 231 bp downstream of the start codon.

Transformation of *N. tabacum*

Binary vectors containing the 35S–S_2-gene constructs and the 35S-GUS construct (Jefferson et al., 1987) were transferred to *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) by triparental conjugation (Fraley et al., 1983) using the helper plasmid pRK2013 (Figurski and Helinski, 1979). *N. tabacum* var Wisconsin 38 plants were transformed using the leaf disc transformation method of Horsch et al. (1985). Plant tissues were cultured in MS medium (Murashige and Skoog, 1962) containing 30 g/L sucrose and 8 g/L agar. *N. tabacum* plants were grown axenically on hormone-free MS medium, and leaves from these plants were used for transformation. Transgenic shoots were regenerated (6 to 8 weeks) on MS medium supplemented with the hormones benzyladenine, 6-aminopurine (1 mg/L), and 3-indoleacetic acid (0.5 mg/L), and the antibiotics cefotaxime (250 mg/L) and kanamycin monosulphate (100 mg/L). Regenerated shoots were excised and transferred to hormone-free MS medium containing the same levels of antibiotics. Any shoots that produced roots on this medium (1 to 3 weeks) were transferred to a glasshouse.

Protein Gel Blot Analysis

Proteins were extracted from plant tissues in a buffer containing 150 mM Tris-HCl, pH 8.5, 5 mM EDTA, 100 mM 2-mercaptoethanol, and

1% (w/v) insoluble polyvinylpyrrolidone (Polyclar AT; BDH Chemicals Ltd., Poole, U.K.). Protein concentrations were determined using the Bradford method (Bradford, 1976) with BSA as a standard. The proteins were size fractionated on 15% SDS-polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose filters (BA 83; Schleicher & Schuell) by electroblotting (Bio-Rad). Loading was checked by staining the filters with Ponceau S (Gelman Instrument Co., Kew, Victoria, Australia). Purified S_2-glycoprotein for control lanes was prepared as described by Jahnen et al. (1989a). The filters were probed with an S_2-peptide-specific polyclonal antibody from sheep (Anderson et al., 1989), and bound antibody was detected by the Amersham biotin-streptavidin system (Amersham International), using procedures recommended by the manufacturer.

Partial Purification of the S_2-Glycoprotein from the Transgenic Plants

Proteins were extracted from 500 styles (includes stigmas and styles) of plants expressing the 35S-genomic S_2 construct and prepared for fast protein liquid chromatography essentially as described by Jahnen et al. (1989a). The proteins were loaded onto a cation exchange column (Mono S 5/5; Pharmacia) that was previously equilibrated in acetate buffer (0.05 M NaOAc, pH 5.0). Bound protein was eluted with a salt gradient (0 to 0.5 M NaCl) in acetate buffer, and material eluting between 0.25 and 0.32 M NaCl was pooled (15 mL), diluted to 30 mL with acetate buffer, then loaded onto the Mono S column that was reequilibrated with acetate buffer. Bound protein was eluted with a pH gradient as follows: buffer A, 0.05 M NaOAc, pH 5; buffer B, 0.05 M Na_2HOP_4, 0.05 M glycine, pH 10.25. Proteins were adsorbed onto the Mono S column in buffer A, washed in 70% buffer A, 30% buffer B, and eluted with a 30-mL gradient to 20% buffer A, 80% buffer B. Material eluting between pH 8.98 and pH 9.89 was pooled, and the solution was neutralized with 0.1 volume of 1 M Tris-HCl, pH 6.8. The proteins were recovered by ammonium sulphate precipitation and quantified using the Bradford (1976) method with BSA as a standard.

Ribonuclease Assays

Proteins were assayed for ribonuclease activity essentially as described by Brown and Ho (1986) with modifications by McClure et al. (1989). A unit of activity was defined as an increase of one A_{260} unit per min in a 1-mL reaction volume. Specific activity was calculated as units per milligram of total protein (Table 1).

Enzymic Removal of *N*-Linked Glycan Chains

The partially purified proteins from the transgenic plants and purified S_2-glycoprotein from *N. alata* were digested with the peptide *N*-glycosidase F (*N*-glycanase; Genzyme Corp., Boston, MA) essentially as described by the manufacturer. The S_2-glycoprotein from *N. alata* was mixed with total soluble protein from styles of untransformed *N. tabacum* at a rate of 6% (w/w). The proteins (12.5 µg of total protein, 0.17 mg/mL) were heated to 100°C for 3 min in 150 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.5% SDS, 0.1 M 2-mercaptoethanol. The solution was cooled to 37°C, and Nonidet P-40 (50% v/v, 1.85 µL) was added. An aliquot (15 µL, for time T = 0 min) was removed, and *N*-glycanase (0.5 units in 2 µL volume) was added to the remaining solution, which was incubated at 37°C. At 10, 30, and 120 min, aliquots were removed (21, 21, and 19 µL, respectively) and analyzed using a protein gel blot.

Immunocytochemistry

Styles from transformed and untransformed plants were sectioned as previously described (Anderson et al., 1986). Sections (8 µm) were preincubated (10 min) in BSA buffer (1% BSA, 1 × PBS [0.5 M NaCl]) with 0.05% Tween 20. Sections were then incubated (1 hr) with S_2-peptide-specific antibody (Anderson et al., 1989) (17 µg/mL) in BSA buffer, washed in 1 × PBS, 0.05% Tween 20, and labeled (1 hr) with rabbit anti-sheep IgG antibody conjugated to 5-nm colloidal gold (Janssen, Belgium). The sections were washed, then silver-enhanced using an enhancement kit (Janssen), and photographed under polarized light.

Dissection of Style Tissues

Twelve styles of flowers at anthesis from plants transformed with the 35S-genomic S_2 construct were dissected using fine scalpels and forceps, in 2 mL of extraction buffer (150 mM Tris-HCl, pH 8.5, 5 mM EDTA, 100 mM 2-mercaptoethanol, and 300 mM sucrose). The cortical, vascular, and epidermal tissues were separated from the transmitting tract and stigmatic cells, and transferred to a fresh tube containing 2 mL of extraction buffer. The buffer containing the transmitting tract and stigmatic cells was filtered through a 30-µm nylon mesh (Nytal; Swiss Screens [Australia] Pty. Ltd.), and the buffer, containing soluble secreted proteins from the styles, was retained. The cellular fractions were rinsed with fresh buffer, and soluble proteins were extracted. Ten *N. alata* genotype S_2S_2 styles were dissected in a similar manner.

RNA Gel Blot Analysis

Tissues for RNA extractions were collected, snap frozen in liquid N_2, and stored at −70°C. Total RNA was extracted as described by McClure et al. (1990). The RNAs were size fractionated in 1.8% agarose-formaldehyde gels (Maniatis et al., 1982) and transferred to a ZetaProbe membrane (Bio-Rad) by wet blotting (Maniatis et al., 1982) in 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate). Hybridizations were performed at 68°C in 1.5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) (Maniatis et al., 1982), 1% SDS, 1% powdered milk, and 4 mg/mL degraded DNA. Final washing conditions were 0.2 × SSPE, 1% SDS at 68°C. The β-glucuronidase (GUS) probe was prepared using a 1.87-kb PstI fragment containing the GUS coding region, isolated from pRAJ260 (provided by Dr. R. Jefferson; Jefferson et al., 1986). Blots containing RNA samples from different organs of transgenic plants were checked to ensure that they were loaded with equal quantities of nondegraded RNA, by hybridization with a ^{32}P-labeled DNA fragment encoding a pea 28S ribosomal RNA (kindly provided by Dr. W. F. Thompson; Jorgensen et al., 1987).

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