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The S-Ribonuclease Gene of *Petunia hybrida* Is Expressed in Nonstylar Tissue, Including Immature Anthers[1]

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To determine the ability of isolated S-locus promoter sequences to direct organ-specific gene expression, we used microprojectile bombardment to introduce chimeric S-allele/β-glucuronidase genes into different tissues of *Petunia hybrida* for transient expression. Histochemical staining showed that S-locus/β-glucuronidase fusions were expressed in pistil, ovary, and petal tissue. No expression of the chimeric genes was detected in leaves or in mature pollen, either by histochemical staining or by fluorescence assays. RNA blot hybridization confirmed that low levels of S-locus mRNA accumulate in petals and ovaries in vivo. Analysis of the expression pattern of S-locus promoter deletions showed that sequences in the immediate vicinity of the TATA box were sufficient to confer qualitatively correct organ-specific expression of β-glucuronidase. To further investigate the potential for S-ribonuclease expression in pollen, we used the polymerase chain reaction to amplify RNA accumulated in developing anthers. These assays demonstrated that mRNA for the S-ribonuclease accumulates to low levels in developing anthers several days prior to corolla opening and pollen anthesis. We discuss these results in light of current models of self-incompatibility.

Gametophytic self-incompatibility, a genetic barrier to inbreeding found in over half the families of angiosperms, is characterized by the ability of styles to discriminate among different pollen tubes on the basis of allelic specificity. Historically, self-incompatibility has been thought to be controlled by a single, multi-allelic locus known as the S-locus (de Nettancourt, 1977; Sims, 1993). In gametophytic self-incompatibility, pollen tubes expressing an S-locus specificity identical to that expressed by the style cease growing approximately one-third of the way down the style transmitting tract, whereas pollen tubes lacking allelic identity with the style continue to grow through the style to the embryo sac, where fertilization occurs.

One characteristic feature of self-incompatibility is the precise pattern of temporal and tissue-specific expression of the S-locus. In previous work (Clark et al., 1990) we demonstrated that temporal expression of the S-locus is directly correlated with the acquisition of the self-incompatibility response and that high levels of S-locus mRNA accumulate preferentially in styles. Cornish et al. (1987) used in situ hybridization to pistils of *Nicotiana alata* to show that S-locus mRNA accumulated in cells of the upper portion of the stylar transmitting tract, as well as in epidermal cells of the ovary placenta. This tissue-specific pattern of expression is an apparent reflection of the cellular role of the S-locus; S-allele-encoded protein accumulates preferentially in those locations where it can interact with elongating pollen tubes.

All of the style-expressed gametophytic S-alleles that have been cloned to date show homology to a class of fungal RNases (Horiuchi et al., 1988; Kawata et al., 1988; McClure et al., 1989; Clark et al., 1990; Ioerger et al., 1991) as well as to related RNases from self-compatible species (Ide et al., 1991; Jost et al., 1991; Taylor et al., 1993). Furthermore, the S-locus proteins have RNase activity in vitro (McClure et al., 1990; Gray et al., 1991; Singh et al., 1991) and have been renamed S-RNases to reflect this function. According to current models (e.g. Haring et al., 1990; Gray et al., 1991; Sims, 1993), interaction of the S-RNase with a proposed receptor, active in the pollen tube, is followed by the uptake of the S-RNase protein into incompatible pollen tubes, degradation of pollen tube RNA, and inhibition of pollen tube growth.

The nature of the proposed pollen S-allele receptor remains unclear. Although previous models of self-incompatibility proposed that the pollen and style products of the S-locus were identical (de Nettancourt, 1977), to date no expression of style-expressed S-locus sequences has been observed in either mature pollen or in germinating pollen tubes (Clark et al., 1990; Haring et al., 1990; Thompson and Kirch, 1992; Sims, 1993). Failure to observe pollen expression of cloned S-allele sequences could result from insufficient sensitivity in detecting rare mRNAs, from expression of the pollen sequences only in response to signals received during pollination in vivo, or from expression of the S-RNase gene sequences in developing pollen. An alternative hypothesis is that the S-locus is complex, with the pollen receptor encoded by a separate gene that is tightly linked to the S-RNase coding sequence (Clark et al., 1990; Thompson and Kirch, 1992; Sims, 1993).

To further investigate the potential for expression of the S-RNase sequences in pollen, and to begin to analyze those

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Abbreviations: CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; LUC, firefly luciferase; MU, 4-methyl umbelliferone; NOS, nopaline synthase; RT-PCR, reverse transcriptase PCR; S-allele, self-incompatibility allele; S-locus, self-incompatibility locus.

sequences regulating the specific developmental expression of the S-locus, we used microprojectile bombardment to introduce chimeric S-allele/GUS (Jefferson et al., 1986, 1987; Jefferson, 1987) genes into plant cells for transient expression assays. We report here that S-allele/GUS fusion genes are expressed in pistil, ovary, and petal tissue but are not expressed at detectable levels in leaves or mature pollen. Sequences immediately adjacent to the TATA box are apparently sufficient to allow qualitative expression of the S-locus in pistils and petals. PCR amplification assays, however, demonstrated that S-RNase sequences are expressed in immature anthers several days before anthesis.

MATERIALS AND METHODS

GUS Fusion Constructs

Chimeric gene fusion constructs between promoter regions of the S_1-RNase and GUS are shown in Figure 1. The initial S_1 promoter/GUS fusion gene (S_1-GUS) was constructed in two steps. First, we constructed a promoterless GUS cloning vector (pUC/GUS) by ligating the structural region (5′ polylinker, GUS coding sequences, 3′ NOS terminator) from pBI101.3 (Jefferson, 1987) to pUC119 (Viera and Messing, 1987) previously digested with restriction enzymes *Hin*dIII and *Eco*RI. The pUC/GUS plasmid was then digested with *Hin*dIII and *Sma*I and ligated to a 1945-bp *Hin*dIII/*Pvu*II genomic DNA fragment containing 5′ flanking sequences of the S_1 gene (Fig. 1). The resulting construct (S_1-GUS, Fig. 1) is a translational fusion having 1905 bp of S_1 5′ flanking DNA, the entire S_1 allele 5′ nontranslated region, and the first five amino acids of the S_1 signal sequence fused in-frame to GUS. A second fusion having S_1 termination sequences replacing the NOS terminator (S_1-3′, Fig. 1A) was constructed by digesting the S_1-GUS fusion with *Sst*I, making the ends flush with the Klenow fragment, then digesting with *Eco*RI and ligating to a 551-bp *Rsa*I-*Eco*RI fragment containing 3′ flanking sequences of the S_1 allele. Several constructs were used as controls for organ-specific expression or for coprecipitation assays. pBI221 (Jefferson, 1987) is a CaMV 35S-GUS fusion construct in pUC19. pDO432 (Ow et al., 1986) is a CaMV 35S-LUC fusion. pLAT52–7 (Twell et al., 1989) and pLAT59–13 (Twell et al., 1990) are fusions between promoter regions of *Lycopersicon esculentum* pollen-specific genes and the GUS and LUC reporters, respectively.

Deletion Constructs

Deletion clones having various amounts of S_1 5′ flanking sequences fused in-frame to GUS were constructed using an exonuclease III kit (Promega). S_1-GUS (or for fine-scale deletions, a 2.65-kb *Pst*I/*Eco*RI subclone of S_1-GUS) was subcloned into polylinker sites of Bluescript SK$^-$ (Stratagene) and exonuclease III deletions were constructed following the supplier's protocol. A fusion gene with a larger (8 kb) 5′ flanking sequence was constructed by digesting an 8.6-kb *Bgl*II genomic subclone in pUC119 with *Sph*I and *Pvu*II and ligating to pUC/GUS digested with *Sph*I and *Sma*I.

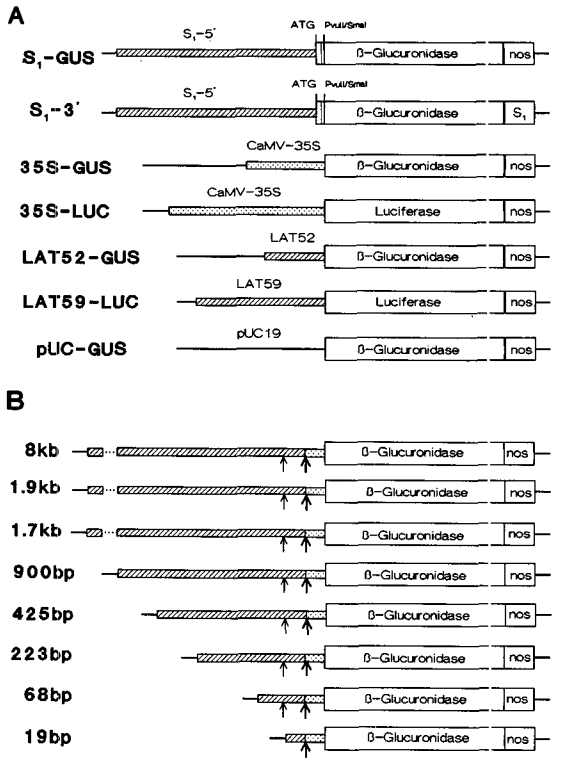

Figure 1. Chimeric gene fusions with GUS and LUC reporter genes. A, S_1 allele fusions and control gene constructs. S_1-GUS, 1.9-kb S_1 allele 5′ flanking sequence fused to GUS. The ATG indicates the position of the S_1 translational start, and the *PvuII*/*SmaI* fusion junction is indicated. S_1-3′, Identical construct to S_1-GUS, except that the NOS termination sequences were replaced with a 551-bp *Rsa*I-*Eco*RI fragment containing S_1 allele 3′ termination sequences. 35S-GUS, pBI221 (Jefferson, 1987), CaMV 35S promoter fused to GUS. 35S-LUC, pDO432 (Ow et al., 1986), CaMV 35S promoter fused to LUC. LAT52-GUS (Twell et al., 1989), tomato LAT52 pollen-specific promoter fused to GUS. LAT59-LUC (Twell et al., 1990), tomato LAT59 pollen-specific promoter fused to LUC. pUC-GUS, promoterless GUS cassette. B, S_1 allele deletion constructs fused to GUS. The large arrows indicate the position of the major mRNA start site, and the small arrows show the position of the minor mRNA start (Clark, 1991). The labels of the individual constructs designate the amount of S_1 promoter sequence upstream of the major mRNA start site. The construct labeled 1.9 kb is identical to the construct labeled S_1-GUS in A.

RNA Isolation, RNA Blots, and RNase Protection

Isolation of total and polyadenylated RNA fractions and RNA electrophoresis methods have been described previously (Clark et al., 1990). For RNase protection assays, a 554-bp *Pst*I fragment spanning the 5′ end of the S_1 coding region was subcloned into Bluescript SK$^-$ (Stratagene), and labeled RNA probes were generated by transcription with T3 and T7 RNA polymerases according to the supplier's protocols (Stratagene). The labeled transcripts were hybridized to total style or petal RNA, digested with RNase, and electrophoresed on sizing gels as described by Sambrook et al. (1989).

Particle Gun Bombardments

DNA (1 μg/shot final concentration) was precipitated onto gold or tungsten (1–3 μm diameter) particles and used for bombardment, according to the method of Klein et al. (1988). Tissue to be used for particle bombardment was excised and placed on nutritive agar plates (Horsch et al., 1988). For bombardment of styles, 25 styles were arranged in a circular pattern with the basal portion of the style inserted into the agar and the stigma raised above the surface of the agar so that particles would penetrate the stigmatic surface at an oblique angle. For pollen bombardment, a piece of Whatman 3MM filter paper was laid onto the agar surface and overlaid with a nitrocellulose membrane, and pollen grains or pollen germinated in vitro (Mulcahy and Mulcahy, 1988) pipetted onto the surface of the membrane. Tissue was placed approximately 10 cm below the barrel of a PDS-1000 particle gun (DuPont) and bombarded under vacuum (20–25 millitorr).

Histochemical Assays of GUS Activity

Following bombardment, tissue samples were allowed to express the introduced constructs for 6 to 24 h under ambient light conditions. For histochemical staining, GUS enzyme activity was localized to individual petunia (*Petunia hybrida*) cells using 5-bromo-4-chloro-3-indolyl glucuronide, according to the method of Jefferson (1987). Samples were photographed using either a Wild M54 microscope or a Zeiss Photomicroscope III. In some cases (see Fig. 2B), specific stained tissues were photographed using the dissecting microscope, then sectioned using a vibratome, and photographed with the compound microscope for visualization of individual stained cells.

Fluorometric Assays of GUS Activity

For fluorometric assays, GUS constructs were precipitated onto particles so that there was 2 μg of DNA per shot. Following bombardment, tissues were allowed to express for 24 h under ambient light conditions prior to extraction. Equal weights of bombarded tissue were frozen in liquid nitrogen, ground to a fine powder, and extracted with 2 mL of buffer (100 mM $NaPO_4$, pH 8.0, 10 mM DTT, 1 mM EDTA, 0.1% Triton X-100) using a tissumizer (Tekmar, Cincinnati, OH). Particulate matter was pelleted by centrifugation and the supernatant was split into aliquots for assays of GUS activity and protein concentration. Protein concentration was measured according to the method of Bradford (1976) using a Bio-Rad protein assay kit. For the fluorogenic GUS assay, 400 μL of crude extract was added to 550 μL of GUS assay buffer (100 mM $NaPO_4$, pH 8, 10 mM DTT, 10 mM EDTA, 0.1% Triton X-100, 22 mg mL^{-1} 4-methyl umbelliferyl glucuronide) and the reaction was incubated at 37°C. Timed aliquots (t = 0, 1, and 2 h) of 300 μL were removed and added to 1.7 mL of 0.2 M $NaCO_3$ to terminate the reaction (the reaction was linear over time for at least 4 h). Fluorescence of the product MU was measured in a Perkin-Elmer LS-5 fluorescence spectrophotometer using an excitation wavelength of 365 nm and a detection wavelength of 455 nm. Calibration of the spectrophotometer was carried out as described (Jefferson, 1987), and raw fluorescence units were converted to pmol MU h^{-1} mg^{-1} protein by setting a 10 nM concentration of MU equal to 1000 fluorescence units.

Co-Precipitations and LUC Activity Measurements

For co-precipitation assays, GUS constructs were precipitated onto particles along with constructs containing either CaMV 35S (Ow et al., 1986) or LAT59 (Twell et al., 1990) promoter sequences fused to the LUC gene. DNAs were precipitated so that 1 μg of each construct was used per shot. Samples were extracted as above and split into three aliquots for determination of protein concentration, GUS activity, and LUC activity. LUC activity was assayed by adding 100 μL of tissue extract to 500 μL of buffer (20 mM Tricine, pH 7.8, 10 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM CoA, 470 μM luciferin, 530 μM ATP) and measuring luminescence using a luminometer with an integration time of 10 s.(Wood, 1990).

PCR Primers

Primers to be used for amplification of S-RNase sequences were designed with the aid of the PCRPlan program of PCGene (IntelliGenetics, Mountain View, CA). Sequences of individual primers are given below; their positions in the S_1-RNase gene are shown in Figure 4 (see "Results") and discussed in the text. Primers used for RT-PCR and nested amplifications were the following:

S_1-P1 5′-ATTTCTGGGTCTTTCGACCACTGGC-3′
S_1-M1 5′-GGTTTCACTCGGGTTACTGACGCG-3′
S_1-P2 5′-AGGTTGTCCGAGACCAGTAATTCCG-3′
S_1-M2 5′-TTTCTCCAGTATAAGTTGATCCCGG-3′
S_3-P1 5′-TGCTCTTTCTCCCGTTAGTGC-3′
S_3-M1 5′-CTGTCTGTTTCATCGCATGTGTAG-3′

RT-PCR Assays

First-strand cDNA was synthesized using 1 μg of total RNA as template for Superscript (BRL) reverse transcriptase. Following first-strand synthesis, RNA template was removed using RNase H (Hu et al., 1991), and cDNA was used as a template for the PCR. Amplifications were performed in a volume of 50 μL (RT-PCR) or 100 μL (nested amplifications) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 200 μM each deoxynucleoside triphosphate, and 1 μM each primer. RT-PCR amplifications used 2 μL of the first-strand cDNA reaction. For control amplifications, either 250 ng of genomic DNA or 5 pg of the indicated cDNA clone was used as template. Reactions were overlaid with 75 μL of mineral oil, then amplified in a Perkin-Elmer model 480 thermocycler using the following program: 94°C, 15 min; (94°C, 1 min; 55°C, 2 min; 72°C 3 min); 4°C soak; 35 cycles total. To allow completion of partially extended chains, the synthesis step was extended for an additional 5 s at each cycle. Products of the PCR reactions were analyzed as described below. Nested amplifications were performed in a manner designed to minimize the possibility of accidental

contamination by style cDNA or cloned DNAs. Aliquots of the primary PCR amplification reaction were electrophoresed on a preparative gel with two lanes between samples to reduce the chances for cross-lane contamination. Stained bands were cut out of the gel using individual sets of sterile instruments, and DNA was isolated using Gene-Clean (Bio 101, La Jolla, CA) and resuspended in 20 μL of 10 mM Tris-HCl, pH 8, 0.1 mM Na_2EDTA. The nested amplification reaction used 2.5 μL of the isolated DNA as template. All PCR reactions were performed using completely separate sets of automatic pipettors, barrier tips, and reagents from those used for analysis of PCR products.

PCR Cloning and DNA Sequence Analysis

A vector for cloning of PCR products was prepared by digesting Bluescript SK^- (Stratagene) with *Eco*RV, then adding a single dT extension using the method of Marchuk et al. (1991). PCR products were ligated directly into this vector and used to transform *Escherichia coli* MV1190. Transformed colonies were screened for insert size, and DNA was prepared for double-stranded sequencing with Sequenase (United States Biochemical). DNA sequence analysis was performed using the University of Wisconsin Genetics Computer Group (Devereux et al., 1984) and PCGene (IntelliGenetics) programs.

Anther Staging and Development

Anthers from defined developmental stages (Clark et al., 1990) were fixed and paraffin embedded according to the procedures of Cox and Goldberg (1988) and Bowman et al. (1991). Anther sections (7–10 μm) were stained with safranin/fast green as described by Jensen (1962).

RESULTS

Construction of S_1-GUS Gene Fusions

Sequence analysis of the S_1 gene region (Clark, 1991) (Fig. 1) identified a *Pvu*II site 15 bp downstream of the translation initiation codon. We used that site to make a fusion construct with the GUS reporter gene (Jefferson et al., 1986, 1987; Jefferson, 1987). This fusion, S_1-GUS (shown in Fig. 1A), contains 1905 bp of S_1 5′ flanking DNA, the entire S_1 allele 5′ noncoding region, and the first five amino acids of the S_1 signal sequence (Clark et al., 1990) fused in-frame to GUS. An alternative construct (S_1-3′ in Fig. 1A), in which the NOS 3′ end was replaced with 3′ flanking sequences from the S_1 allele, was constructed by digesting the S_1-GUS construct with *Sst*I and *Eco*RI and replacing the NOS 3′ end with a 551-bp *Rsa*I-*Eco*RI fragment containing 3′ flanking sequences of the S_1 allele. Several deletion clones, having from 8 kb to 19 bp of 5′ flanking DNA, were constructed as described in "Materials and Methods" and are discussed in the sections below.

Transient Expression of S_1-GUS Chimeric Genes in Pistil Tissue

In previous work we had shown that S-locus mRNAs accumulate to 0.65% to 1.5% of the mRNA mass of styles, and to a far lesser extent in ovaries (Clark et al., 1990). To determine if 5′ flanking sequences of the S_1 gene could be used to achieve transient expression of genes in petunia tissues, we bombarded either detached styles or intact pistils (excised below the pedicel) with the S_1-GUS construct (Fig. 1A) and assayed for expression by histochemical staining. Stained cells (Fig. 2, A and B) were observed following bombardment of the stigma in areas that corresponded to tissue contiguous with the style transmitting tract and that had previously been shown to express S-alleles in vivo (Cornish et al., 1987). No staining was observed when a promoterless GUS plasmid (pUC-GUS in Fig. 1A) or tungsten particles alone were used for bombardment (Table I).

Organ-Specific Expression of the S_1-GUS Fusion Gene

Our earlier experiments (Clark et al., 1990) had demonstrated expression of S-locus sequences in styles and ovaries but not in leaves or in mature or germinating pollen. To determine if expression of the S_1-GUS construct was limited to cells of the pistil, this same S_1-GUS construct was used in bombardments of ovaries, petals, leaves, and pollen. We observed expression of the S_1-GUS fusion in cells of the ovary wall (Fig. 2C) and in petals (Fig. 2D), but we never observed expression of the S_1-GUS fusion in leaves or pollen. Bombardment of leaves with CaMV 35S-GUS (pBI221; Jefferson, 1987) or of pollen with LAT52-GUS (a fusion between the *L. esculentum* pollen-specific gene LAT52 and GUS; Twell et al., 1989) routinely resulted in the observation of blue-stained cells (Fig. 2, E and F).

To be certain that the failure to observe expression of the S_1-GUS fusion genes in either leaf or pollen cells did not result simply from inefficient DNA delivery or a low frequency of expression, we co-precipitated the S_1-GUS construct onto particles along with either CaMV 35S-LUC (pDO432; Ow et al., 1986) or pLAT59-LUC (LAT59-LUC; Twell et al., 1990) prior to bombardment. The CaMV 35S-LUC construct consistently gave LUC activity levels well above background (Table I) in co-bombardments of leaves, whereas the S_1-GUS, S_1-3′, or S_1 (1.7 kb)-GUS constructs (see Fig. 1B) never showed GUS activity levels over background (Table I). Similarly, the LAT59-LUC construct showed strong expression in co-bombardments of pollen, whereas S_1 promoter sequences never gave GUS activity levels over background in this tissue (Table I). By contrast, in experiments where petals or pistils were co-bombarded with S_1-GUS and pDO432, GUS activity was always observed whenever LUC activity was significantly above background level (Table I).

Petal and Ovary Expression of S-Locus Sequences

Although we had previously observed low-level expression of the S_1 allele in ovary tissue (Clark et al., 1990), we had not detected an equivalent level of expression in petals. To determine if the expression we observed in petals might result from foreign 3′ flanking sequences that conferred enhanced mRNA stability, we replaced the NOS 3′ end of S_1-GUS with 3′ flanking sequences from the S_1 allele. This replacement

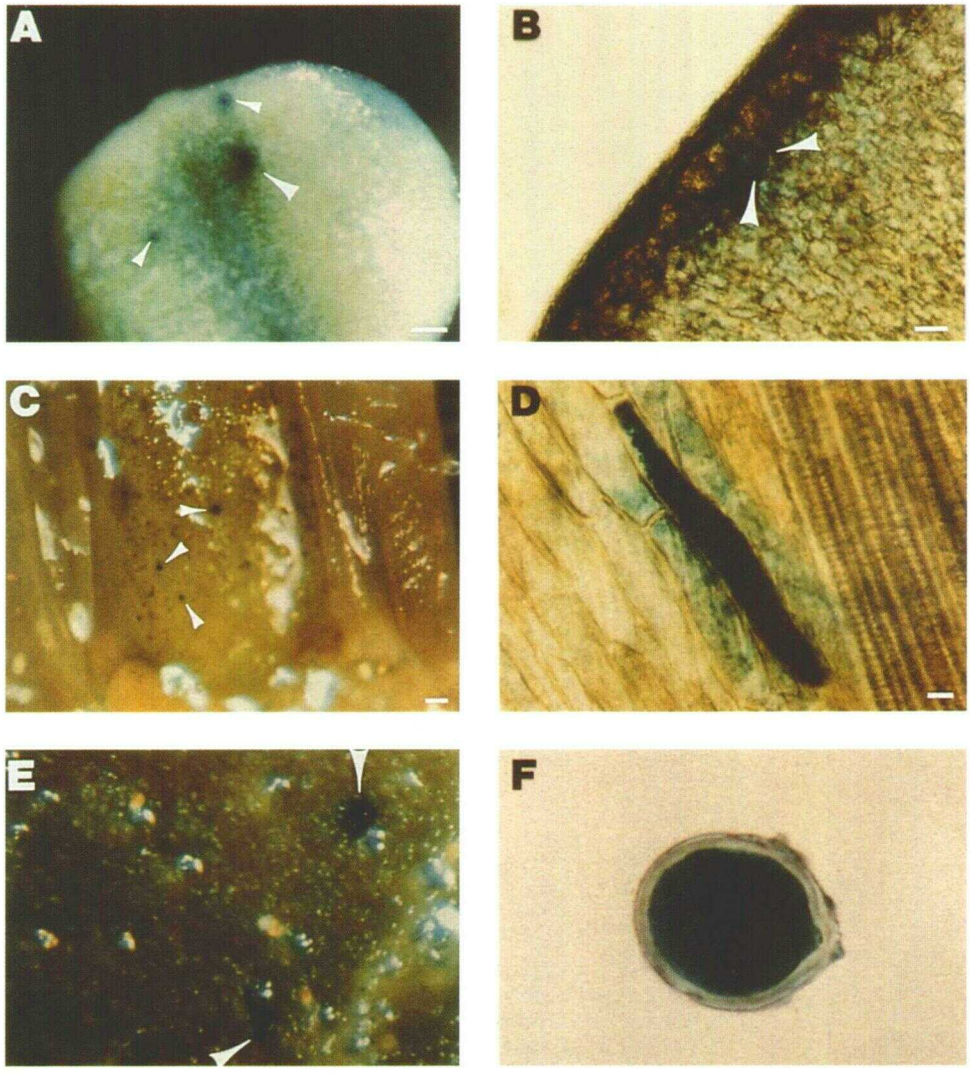

Figure 2. Histochemical staining showing transient expression of S_1 allele/GUS fusions following particle bombardment of different petunia tissues. Arrowheads indicate the positions of stained cells. A, Photograph of stigmatic surface of whole style showing GUS expression in stigmatic cells contiguous with the style transmitting tract. Bar = 250 μm. B, Section through upper portion of the stigma of another style. A stained style (similar to that shown in A) was sectioned on a vibratome in the region of GUS expression, and the section was mounted and photographed with a compound photomicroscope. The arrowheads show a cell expressing the S_1-GUS fusion gene. Bar = 20 μm. C, S_1-GUS expression in cells of the ovary. Intact pistils, including the ovary, were bombarded from the side, allowed to express for 24 h, and stained for GUS activity. Bar = 250 μm. D, S_1-GUS expression in petal tissue. Petals were excised from the corolla, bombarded, and stained. The photograph shows an individual petal cell displaying transient expression. Bar = 20 μm. E, Control expression of a CaMV 35S-GUS fusion in leaf. Two cells, including an individual trichome, are staining for GUS activity. Bar = 250 μm. F, Individual pollen grain showing expression of the LAT52-GUS gene construct. Bar = 20 μm.

had no effect on expression of the S_1 fusion in petals. Similarly, differing extents of 5′ flanking region had no qualitative effect on the expression of the S_1-GUS fusion in petals (Table I).

To be certain that the S_1-GUS expression observed in petals reflected in vivo rather than ectopic expression of the S_1 allele in this tissue, we hybridized sensitive RNA blots of S_1S_1 petal RNA with an S_1 allele cDNA clone, pS1B (Clark et al., 1990). We detected a low level of accumulation of an mRNA (Fig. 3A) in petals that was similar in size (900 nucleotides) to the S-locus mRNA previously observed in styles and ovaries. Filter counts and scans of quantitative slot blots indicated that the level of accumulation of this mRNA was approximately 2-fold lower than that seen in ovaries and at least 2500-fold lower than the level of accumulation seen in styles (data not shown). To determine if the petal sequences were transcribed from the same gene as that expressed in styles, we performed RNase protection mapping of the petal sequence using a cloned fragment that had been previously used to map the 5′ end of the style mRNA. Two bands were

Table I. *GUS activity following bombardment with different gene constructs*

Tissues showing GUS histochemical staining following bombardment are indicated by plus (+++) signs, those not showing staining are indicated by dashes (—). Fluorescence activity measurements from GUS/LUC co-bombardments are shown where those assays were performed, with the number of replicates given in parentheses. GUS activity units are given as pmol MU h^{-1} mg^{-1} protein. LUC units are given as luminometer units mg^{-1} protein. Endogenous background activity of nonbombarded tissue has been subtracted from all data points. This results in artificially negative values for some constructs. nd, Not determined.

GUS Construct	Stigma		Leaf		Pollen		Petal		Ovary
	GUS	LUC (35S-LUC)	GUS	LUC (35S-LUC)	GUS	LUC (LAT59-LUC)	GUS	LUC (35S-LUC)	GUS
S_1-8 kb	+++		—		—		+++		+++
	1.20 ± 0.24 (4)	0.11 ± 0.05		nd	−0.92 ± 0.16 (4)	2.54 ± 1.53		nd	
S_1-GUS	+++		—		—		+++		+++
	1.09 ± 0.10 (3)	0.34 ± 0.15	−0.04 ± 0.02 (2)	0.12 ± 0.06		nd	0.41 ± 0.25 (2)	0.41 ± 0.20	
S_1-3′	+++		—				+++		
	1.25 ± 0.21 (4)	0.27 ± 0.13	−0.05 ± 0.03 (5)	0.46 ± 0.36	nd	nd		nd	nd
S_1-1.7 kb	+++		—				+++		
		nd	−0.03 ± 0.07 (3)	0.60 ± 0.23	nd	nd		nd	nd
S_1-900	+++	nd	—	nd	nd	nd	+++	nd	nd
S_1-425	+++	nd	—	nd	nd	nd	+++	nd	nd
S_1-223	+++		—				+++		
	1.22 ± 0.28 (4)	0.20 ± 0.13		nd	nd	nd		nd	nd
S_1-69	+++		—				+++		
	0.50 ± 0.21 (6)	0.09 ± 0.07		nd	nd	nd		nd	nd
S_1-19	—		—				—		
	0.02 ± 0.05 (2)	0.08 ± 0.02		nd	nd	nd		nd	nd
35S-GUS	+++		+++		nd	nd	+++		+++
		nd	0.30 ± 0.20 (7)	0.31 ± 0.33	nd	nd	0.94 ± 0.18 (2)	0.49 ± 0.02	
LAT52-GUS	nd	nd	nd	nd	+++	nd	nd	nd	nd
pUCGUS	—	nd	—	nd	—	nd	—	nd	—

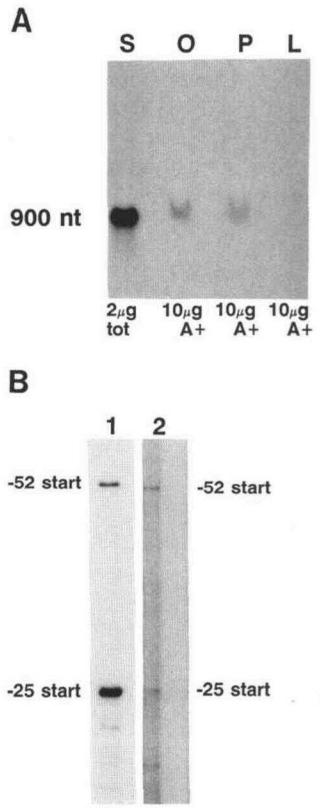

Figure 3. A, RNA blot hybridization showing the relative accumulation of mRNA encoding the S_1-RNase in different petunia organs. Lane S, Style RNA, 2 μg of total RNA. Lane O, Ovary RNA, 10 μg of poly(A)$^+$ RNA. Lane P, Petal RNA, 10 μg of poly(A)$^+$ RNA. Lane L, Leaf RNA, 10 μg of poly(A)$^+$ RNA. Exposure time was 48 h. B, RNase protection mapping of the 5′ end of the S_1 mRNA. Lane 1, Style RNA (5 μg of total RNA, overnight exposure). Lane 2, Petal RNA (40 μg of total RNA, 7-d exposure). The protected bands labeled −25 start and −52 start represent the number of bases upstream of the AUG initiation codon.

protected (Fig. 3B) that were identical in size to those protected in stylar tissue, indicating that the petal and style S-mRNAs were highly similar and were probably identical.

Sequences Immediately Adjacent to the TATA Box Are Sufficient to Allow Qualitative Expression of the S-Locus

To begin to analyze those sequences required for the correct developmental expression of the S-locus, we constructed deletion clones having variable amounts of S_1 5′ flanking sequence fused to the GUS reporter gene (Fig. 1B). To determine the minimal amount of sequence necessary for qualitative expression of the S-locus, we bombarded either petals or styles with the deletion clones and assayed for GUS activity by histochemical staining. Constructs having 8 kb, 1.9 kb, 1.7 kb, 900 bp, 425 bp, 320 bp, 223 bp, and 69 bp of 5′ flanking DNA all showed positive histochemical staining in style and petal tissue following bombardment (Figs. 1 and 2; Table I; data not shown). A construct having only 19 bp of 5′ flanking DNA and lacking a TATA box, however, showed no expression by histochemical staining (Table I). Fluorescence activity measurements of bombarded styles (Table I) gave results qualitatively identical to the histochemical data. Because the construct with 69 bp of flanking DNA was expressed in styles and petals but not in leaves, these results suggest that sequences within close proximity to the mRNA start are sufficient to confer qualitative regulation of S-locus expression.

RT-PCR Assays Show That S-RNase mRNA Accumulates in Immature Anthers

To investigate whether the S-RNase gene might be expressed at a stage of pollen development earlier than we had examined, we used a PCR assay to identify the presence of S_1 mRNA in developing anthers. Total RNA isolated from pooled anthers from developmental stages 4 to 6 (Table II) was used as a template for cDNA synthesis. Following RNase-H digestion to remove residual RNA template, the cDNA was used as a template for the amplification of S-RNase sequences. The locations of the primers used for amplification are shown in Figure 4.

When primers P1 and M1 (see Fig. 4 and "Materials and Methods") were used for RT-PCR amplifications, a band of

Table II. *Floral bud developmental stages*

DPA[a]	Floral Bud Length	Anther Developmental State
9	0.5 cm	Tapetum intact and multinucleate; microspores uninucleate and elongate
7	0.70 to 0.75 cm	Tapetum intact, pollen uninucleate with occasional binucleate grains
6	0.9 to 1.1 cm	Tapetum beginning to degrade; pollen a mixture of uninucleate and binucleate; pollen cytoplasm beginning to stain
5	1.3 to 1.5 cm	Tapetum degrading; pollen binucleate; pollen cytoplasm stained with many small vacuoles
4	1.8 to 2.0 cm	Tapetum degraded, pollen binucleate with strongly staining cytoplasm, many small vacuoles; corolla uncolored
3	2.5 to 3.0 cm	Tapetum gone; pollen cytoplasm highly vacuolate and less densely stained; interlocular wall broken down; corolla beginning to color
2	3.5 to 4.0 cm	Pollen mature, highly vacuolate with prominent exine; strong coloration in corolla
1	4.5 to 4.9 cm	Pollen mature, corolla highly colored and twisted; corolla beginning to open
0	Mature flower	Corolla open, pollen dehisced

[a] DPA, Days prior to anthesis (from Clark et al., 1990).

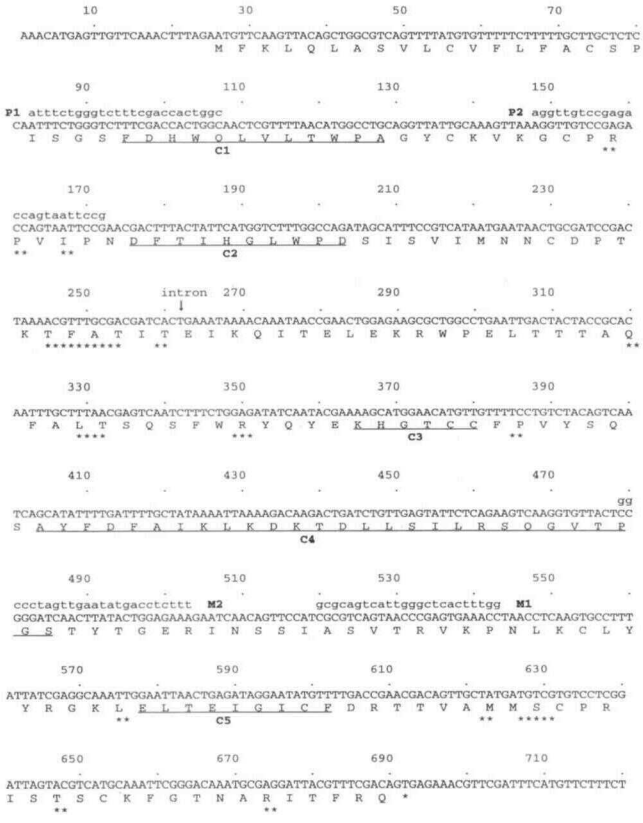

Figure 4. S_1 gene sequence and location of PCR primers used for primary and nested amplifications. The figure shows the DNA sequence corresponding to the S_1 mRNA along with the amino acid sequence of the S_1-RNase. Binding sites for the PCR primers P1, M1, P2, and M2 (see text) are indicated by lowercase letters above the DNA sequence. Underlined amino acid regions labeled C1 to C5 are highly conserved across all S-alleles (see Sims, 1993). Amino acid residues indicated by an asterisk (*) are highly variable across all S-alleles (Sims, 1993). The location of the 118-bp intron is labeled and indicated by an arrow.

approximately 460 bp was amplified from S_1S_1 style RNA and a weak band of similar size was amplified from S_1S_1 immature-anther RNA (Fig. 5A, lanes 1 and 3). A band of identical size was amplified from the control pS1cDNA-clone template (lane 7), whereas a larger band of approximately 580 bp was amplified from S_1S_1 genomic DNA (lane 8). No amplification was observed from S_1S_1 mature-anther (lane 2), germinated-pollen (lane 4), or leaf RNA (lane 5), nor was amplification observed when primers specific for the S_3 allele were used (Fig. 5A, lane 6; Table III). To determine if these amplified bands were specifically derived from S_1 allelic sequences, we performed a nested amplification using an internal set of primers (P2 and M2, Fig. 4). Figure 5B shows that reamplification using the nested primers produces bands of approximately 360 bp in size for both the style and immature-anther bands (lanes 2 and 3). This amplified band is the same size as that predicted from the sequence of the S_1-allele and migrates at the same gel position as a control band amplified from the pS1BcDNA clone (not shown).

A final proof of the identity of the amplified band was obtained by cloning the reamplified immature-anther product into Bluescript SK$^-$ and sequencing. Figure 6 shows the alignment of the DNA sequence from two independent PCR clones with that of the S_1 mRNA coding region. The sequence of both of these clones is identical to that for this region of the S_1 mRNA. Because the cloned region spans the location of the single intron and includes hypervariable regions V2

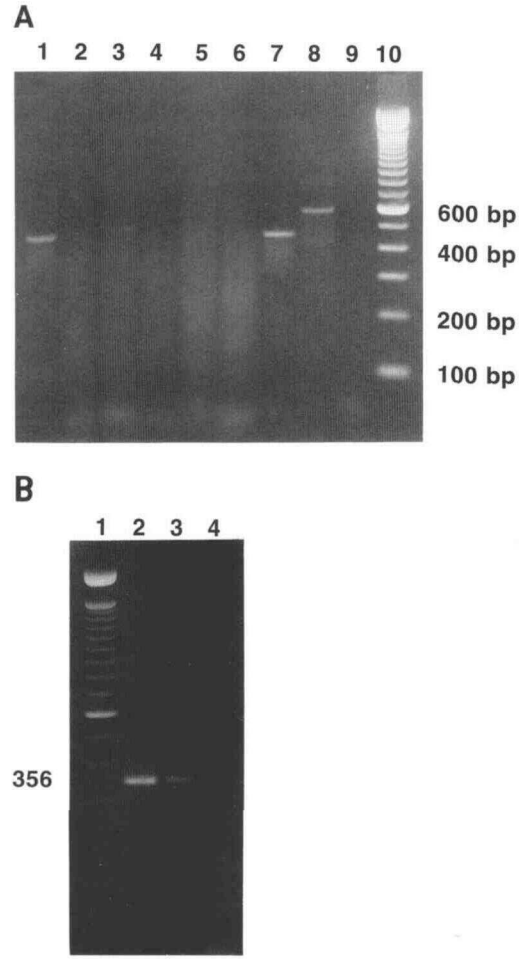

Figure 5. PCR amplifications of S_1 RNA sequences. A, Primary (RT-PCR) amplification using S_1-allele primers P1 and M1 (lanes 1–5, 7–9) or S_3-specific primers (lane 6). Template DNAs were either products of first-strand reverse-transcription reactions (lanes 1–6) or control DNAs (lanes 7 and 8). Lane 1, Style (stage I). Lane 2, Mature anther (stage 1). Lane 3, Immature anther (pooled stages 4–6). Lane 4, Germinated pollen. Lane 5, Leaf. Lane 6, Style, S_3 primer. Lane 7, pS1B cDNA clone. Lane 8, S_1S_1 genomic DNA. Lane 9, Style, reverse-transcriptase left out of cDNA synthesis reaction. Lane 10, DNA size marker (BRL 100-bp ladder). For unknown reasons, the migration of the DNA sample in lane 3 appears to be slightly retarded compared with other lanes. This effect was not seen in other RT-PCR or 3′-rapid amplification of cDNA ends PCR experiments using the same initial templates; in those experiments immature anther and style PCR products co-migrated exactly (data not shown). B, Nested amplification using primers P2 and M2. Lane 1, DNA size marker (BRL 100-bp ladder). Lane 2, Style. Lane 3, Immature anther. Lane 4, Immature anther, S_3 primer.

Table III. *Summary of RT-PCR amplification data*

The table shows the results of RT-PCR amplifications with different primer and template combinations. Except where indicated, all templates were first-strand cDNA reverse-transcribed from the named template RNA. Nested amplifications refer to experiments using band-isolated fragments from the primary RT-PCR reactions (see text). No RT, Reverse transcriptase omitted from cDNA reaction with style RNA as template. A plus (+) sign indicates that amplification was observed; the size of the amplified band is given in parentheses. A minus (−) sign indicates that no amplification was observed. nd, Not determined.

Primer	S_1S_1 Style	S_1S_1 Style Nested	S_1S_1 Immature Anther	S_1S_1 Immature Anther Nested	S_1S_1 Mature Anther	S_1S_1 Germ. Pollen	S_1S_1 Leaf	pS1 cDNA Clone	S_1S_1 Genomic DNA	pS3 cDNA Clone	S_3S_3 Genomic DNA	No RT	No RNA
P1 + M1	+ (461)	nd	+ (461)	nd	−	−	−	+ (461)	+ (579)	−	−	−	−
P2 + M2	nd	+ (356)	nd	+ (356)	nd	nd	nd	+ (356)	+ (474)	nd	nd	−	−
S3 specific	−	−	−	−	nd	nd	nd	−	−	+ (594)	+ (695)	−	−

and V3 (Sims 1993), these data show that the PCR products are derived from S_1 mRNA sequences and not from either genomic DNA or a closely related gene. Together these data indicate that the S_1 gene is expressed in immature anthers and that the RNA product is identical to that found in styles.

DISCUSSION

Organ-Specific Expression of Gene Fusions

Both the histochemical and fluorometric assays shown above (Fig. 2; Table I) demonstrated that flanking sequences of the S_1 allele could direct specific expression of GUS in styles, ovaries, and petals but not in leaves or mature pollen. The expression of S-allele/GUS fusion genes in petals was initially surprising, because we had not previously observed expression of the S-locus in this organ system (Clark et al., 1990). High-sensitivity RNA blot hybridizations along with RNase protection assays (Fig. 3) confirmed the expression of S-alleles in petals and showed that the same gene was expressed in petals and styles. The ability to use GUS fusions to detect such low-level expression—we estimate the S-locus mRNA prevalence in petals to be approximately 3×10^{-4}% of the mRNA mass, a level equivalent to one to two transcripts per cell (Goldberg et al., 1978; Clark et al., 1990)—is both an illustration of the sensitivity of this assay and an additional confirmation that our inability to see S-gene expression in mature or germinating pollen does not result from a lack of sensitivity in assaying for low levels of expression. It is unclear whether the vast difference in the accumulation of mRNA encoding the S_1-RNase in styles, relative to that in petals and ovaries, can be explained by quantitative differences in transcription alone. Previously, Walling et al. (1986) demonstrated that a 500- to 10,000-fold difference in accumulation of different mRNAs in developing soybean cotyledons was correlated with only a 1- to 40-fold difference in the rate of transcription. It seems probable that posttranscriptional processes, including mRNA stability, may be important contributors to the vast difference in S_1-RNase accumulation in different organs.

The results of the RNase protection assay (Fig. 3B) strongly suggest that the mRNA accumulating in ovary and petal cells is identical to that in styles. The RNA blot shown in Figure 3A, however, appears to show petal and ovary S_1-mRNA migrating slightly more slowly than the style mRNA. Al-

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                10         20         30         40         50         60
S1mRNA  AAACATGAGT TGTTCAAACT TTAGAATGTT CAAGTTACAG CTGGCGTCAG TTTTATGTGT

                70         80         90        100        110        120
S1mRNa  TTTTCTTTTT GCTTGCTCTC CAATTTCTGG GTCTTTCGAC CACTGGCAAC TCGTTTTAAC

               130        140        150        160        170        180
S1mRNa  ATGGCCTGCA GGTTATTGCA AAGTTAAAGG TTGTCCGAGA CCAGTAATTC CGAACGACTT
PCRant1                            AGG TTGTCCGAGA CCAGTAATTC CGAACGACTT
PCRant2                            AGG TTGTCCGAGA CCAGTAATTC CGAACGACTT

               190        200        210        220        230        240
S1mRNa  TACTATTCAT GGTCTTTGGC CAGATAGCAT TTCCGTCATA ATGAATAACT GCGATCCGAC
PCRant1 TACTATTCAT GGTCTTTGGC CAGATAGCAT TTCCGTCATA ATGAATAACT GCGATCCGAC
PCRant2 TACTATTCAT GGTCTTTGGC CAGATAGCAT TTCCGTCATA ATGAATAACT GCGATCCGAC

               250        260    270        280        290        300
S1mRNa  TAAAACGTTT GTGACGATCA CTGAAATAAA TCAAATAACC GAACTGGAGA AGCGCTGGCC
PCRant1 TAAAACGTTT GTGACGATCA CTGAAATAAA TCAAATAACC GAACTGGAGA AGCGCTGGCC
PCRant2 TAAAACGTTT GTGACGATCA CTGAAATAAA TCAAATAACC GAACTGGAGA AGCGCTGGCC

               310        320        330        340        350        360
S1mRNa  TGAATTGACT ACTACCGCAC AATTTGCTTT AACGAGTCAA TCTTTCTGGA GATATCAATA
PCRant1 TGAATTGACT ACTACCGCAC AATTTGCTTT AACGAGTCAA TCTTTCTGGA GATATCAATA
PCRant2 TGAATTGACT ACTACCGCAC AATTTGCTTT AACGAGTCAA TCTTTCTGGA GATATCAATA

               370        380        390        400        410        420
S1mRNa  CGAAAAGCAT GGAACATGTT GTTTTCCTGT CTACAGTCAA TCAGCATATT TTGATTTTGC
PCRant1 CGAAAAGCAT GGAACATGTT GTTTTCCTGT CTACAGTCAA TCAGCATATT TTGATTTTGC
PCRant2 CGAAAAGCAT GGAACATGTT GTTTTCCTGT CTACAGTCAA TCAGCATATT TTGATTTTGC

               430        440        450        460        470        480
S1mRNa  TATAAAATTA AAAGACAAGA CTGATCTGTT GAGTATTCTC AGAAGTCAAG GTGTTACTCC
PCRant1 TATAAAATTA AAAGACAAGA CTGATCTGTT GAGTATTCTC AGAAGTCAAG GTGTTACTCC
PCRant2 TATAAAATTA AAAGACAAGA CTGATCTGTT GAGTATTCTC AGAAGTCAAG GTGTTACTCC

               490        500        510        520        530        540
S1mRNa  GGGATCAACT TATACTGGAG AAAGAATCAA CAGTTCCATC GCGTCAGTAA CCCGAGTGAA
PCRant1 GGGATCAACT TATACTGGAG AAA
PCrant2 GGGATCAACT TATACTGGAG AAA

               550        560        570        580        590        600
S1mRNa  ACCTAACCTC AAGTGCCTTT ATTATCGAGG CAAATTGGAA TTAACTGAGA TAGGAATATG

               610        620        630        640        650        660
S1mRNa  TTTTGACCGA ACGACAGTTG CTATGATGTC GTGTCCTCGG ATTAGTACGT CATGCAAATT

               670        680        690        700        710        720
S1mRNa  CGGGACAAAT GCGAGGATTA CGTTTCGACA GTGAGAAACG TTCGATTTCA TGTTCTTTCT

               730        740        750        760
S1mRNa  TTCTAATTTT ATGCAGAGTA TAATAAAGGA GGTTTTACAC TGT
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Figure 6. Alignment of DNA sequence of cloned PCR products with S_1 mRNA coding region. Products of PCR amplification with nested primers (Fig. 5B, lane 3) were cloned into a Bluescript SK^- (Stratagene) T vector (Marchuk et al., 1990) for DNA sequencing. The DNA sequence of two independent clones (opposite orientation in SK^-) is shown aligned with the DNA sequence of the S_1 mRNA coding region. Underlined sequences correspond to the nested PCR primers used for amplification (cf. Fig. 4). The arrow indicates the location of the intron in S_1 genomic DNA.

though this difference could be due to the different amounts of RNA loaded in the different lanes [2 μg of total RNA versus 10 μg of poly(A)$^+$], it is also possible that S-mRNA in ovary and petal differ from the style mRNA in the degree of polyadenylation (see McClure et al., 1993). Also, even though we had previously observed a low level of S-allele mRNA in ovaries by blot hybridization (Clark et al., 1990), expression in the ovary wall was unexpected, because other workers had indicated that S-allele expression in ovaries was limited to epidermal cells of the placenta (Cornish et al., 1987) along with the ovule wall (Anderson et al., 1989). It is possible that a very low level of expression of S-RNase in ovary walls might not have been seen in these previous studies because of insufficient sensitivity of detection. Finally, because the probe used for the RNase protection spans residues encoding the complete signal peptide, it seems probable that the S-RNase expressed in ovaries and petals, like the S-RNase expressed in styles (Anderson et al., 1989), accumulates in extracellular spaces.

Taylor et al. (1993) have shown that mRNAs with homology to *P. hybrida* and *N. alata* S-RNases are expressed in flowers of *Arabidopsis thaliana*. Other related RNase sequences are found in tomato (Jost et al., 1991), *Momordica charantia* (Ide et al., 1991), and fungi (Horiuchi et al., 1988; Kawata et al., 1988). The S-RNases seem clearly to be related to a larger family of RNase proteins that have various roles in different plants and plant tissues. It seems probable that the S-RNases, the related fungal RNases, and the *Arabidopsis RNS* RNase all evolved from a common ancestral protein, with the S-RNases gradually taking on their present role in pollen rejection. It is possible that the petal-expressed S-RNase may play a role in senescence. RNase activity has been reported to be associated with petal senescence (Baumgartner et al., 1975), and Taylor et al. (1993) demonstrated that RNS2 expression increased in senescing leaves and petals of *Arabidopsis*. When we measured S_1 mRNA levels in senescing petals (3–4 DAF), we observed a small increase (1.5×) in expression above the level found in nonsenescing (0 DAF) petals (R. Clark, unpublished results).

Sequences Required for Regulation of S-Allele Gene Expression

Detailed dissection of plant promoters (e.g. Benfey and Chua, 1989; Gilmartin et al., 1990) has shown that multiple *cis*-acting regulatory sequences may act in concert to define the final regulatory properties of a particular gene. At least two broad classes of elements have been characterized: those necessary for qualitative expression of a gene in a particular cell type or tissue and those conferring quantitative modulation of transcriptional levels. The deletion analyses presented here showed that 69 bp of 5′ flanking DNA were sufficient to confer a qualitatively normal organ-specific expression pattern by histochemical staining. Thus, at least for transient expression assays, sequences close to the TATA box are sufficient to allow qualitative expression of the S_1-RNase. It is important to note that the transient assays reported here cannot be interpreted to reflect quantitative regulation of S-locus expression. Bombardments of stigmatic secretory tissue are quite inefficient compared to bombardments of other tissues, owing to the physical properties of the wet stigma in petunia. Furthermore, because Murfet et al. (1992) observed slightly different expression patterns between 35S-GUS and 35S-*N. alata*-S_2 constructs in transgenic plants, it is possible that internal S-RNase gene sequences may affect quantitative expression of the S-locus.

Pollen-Expressed Component of Self-Incompatibility

Previous models of self-incompatibility proposed that the pollen and style products of the S-locus were identical (de Nettancourt, 1977). However, no expression of style-expressed S-locus sequences could be observed in either pollen or in germinating pollen tubes (Clark et al., 1990; Haring et al., 1990; Thompson and Kirch, 1992; Sims, 1993). This failure to observe mature-pollen expression of cloned S-allele sequences led to proposals that the pollen-S receptor was encoded by a tightly linked but separate gene sequence from that encoding the style-expressed S-allele (Clark et al., 1990; Thompson and Kirch, 1992; Sims, 1993). Data presented here demonstrate that S-RNase sequences identical to the stylar RNA do in fact accumulate in immature anther tissue. It is highly unlikely that the amplification we observed resulted from contaminating genomic DNA or cloned sequences. Both the initial primers and the nested primers were designed to hybridize to regions on either side of the 118-bp intron (Fig. 4). The size of the amplified bands, as well as the DNA sequence obtained, indicate that the products are derived from mRNA and not from hypothetical contaminating genomic DNA (compare lanes 1, 3, 7, and 8 in Fig. 5A). Amplification was observed only in reactions using style RNA or RNA from immature anthers; no amplification was observed from mature anthers, pollen germinated in vitro, or leaves. Furthermore, amplification from style RNA or immature anther RNA was not seen in the absence of reverse transcriptase or RNA template. Finally, no amplification was observed when primers specific to the S_3 allele were used, indicating that amplification is specific to the S_1 allele.

Although we would anticipate that this accumulation is pollen specific, to date we have been unable to demonstrate if that is indeed the case. In situ hybridizations with digoxigenin-labeled S-RNase probes to sections of anthers at different developmental stages have thus far failed to detect the accumulation of RNA sequences above the level of background noise. This result is likely due to a very low-level accumulation of the mRNA and the relative insensitivity of the in situ hybridization assays. In a recent report, Dodds et al. (1993) indicated that the S_2 and S_6 alleles of *N. alata* are expressed in immature pollen.

The detection of S-RNase expression in immature anthers of *P. hybrida* (this report) and in immature pollen of *N. alata* (Dodds et al., 1993) is consistent with the traditional one-gene model of S-RNase action (de Nettancourt, 1977; Sims, 1993). Conversely, the self-incompatibility response in pollen remained unaffected in experiments (Lee et al., 1994) that used antisense technology to demonstrate a correlation between decreased S-RNase expression in style and a conversion of *Petunia inflata* from self-incompatibility to self-fertility. Therefore, the role of S-RNase expression in pollen is still unclear. Expression of the S-RNase in developing pollen is

unlikely to result in degradation of pollen RNA, because any protein produced is most probably secreted, similar to the style S-RNase. In this regard, Dodds et al. (1993) reported that the S-RNase protein was localized to the intine of immature pollen of *N. alata*, but (probably for technical reasons) could not be detected in mature pollen.

Current models of gametophytic self-incompatibility (Haring et al., 1990; Gray et al., 1991; Sims, 1993) propose that S-RNase protein is specifically imported into incompatible pollen tubes, with resultant degradation of pollen tube RNA and inhibition of pollen tube growth. If the one-gene model of gametophytic self-incompatibility is correct, then interaction of the style S-RNase and a matching "pollen-receptor" S-RNase should lead to the specific uptake of S-RNase. It is still difficult to understand, however, why in a one-component recognition system, matching of S-alleles (possibly via dimerization; de Nettancourt, 1977) would lead to specific pollen-pistil interaction without any effect of a similar protein-protein interaction in styles alone.

The alternative model would suggest that low-level expression of the S-RNase in developing pollen, perhaps like the similar low-level expression in petals, represents either a different role for the S-RNase in this tissue or a nonsignificant level of basal expression. According to this model, the pollen-S component would be encoded by a tightly linked gene that is separate from that for the S-RNase (Clark et al., 1990; Thompson and Kirch, 1992; Sims, 1993). Okuley and Sims (1991) and Okuley (1991) reported preliminary data suggesting that a sequence region, closely linked to the S_1-RNase, is expressed in pollen, and experiments to investigate that result further are in progress. Recent experiments reported by Lee et al. (1994) and Murfet et al. (1994) have provided direct evidence that style expression of S-RNase is sufficient for pollen rejection. It should now be possible to use in vitro mutagenesis to identify S-RNase protein domains required for recognition, and to determine if alterations of the S-RNase protein affect S-locus function in pollen.

A full resolution of the one-gene/two-gene model of S-RNase action may require experiments to eliminate S-RNase expression in developing anthers or the investigation of pollen-part mutants. In this regard, preliminary RT-PCR experiments (T.L. Sims, unpublished data) indicate that the S_1 primers P1 and M1 used in this work can also be used to amplify RNA sequences from styles of a *P. hybrida* pollen-part mutant (Sims, 1993). We are at present attempting to determine if S-RNase sequences are expressed in developing anthers of this line.

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