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Sequence Variability and Developmental Expression of S-Alleles in Self-Incompatible and Pseudo-Self-Compatible Petunia

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We investigated the structure and expression of three S-alleles of *Petunia hybrida* in self-incompatible varieties and in a pseudo-self-compatible line in which the self-incompatibility response is defective. Comparison of derived amino acid sequences from different gametophytic S-alleles revealed a pattern of sequence conservation and variability that was highly nonrandom. In self-incompatible varieties, petunia S-locus mRNA accumulates preferentially in styles during the transition from bud self-compatibility to self-incompatibility. S-Allele sequences homologous to the cloned S_1 allele were present in a pseudo-self-compatible variety, and were expressed at levels indistinguishable from those observed in a self-incompatible line homozygous for the S_1 allele. Taken together, our data indicate that (1) limited sequence differences may confer allelic specificity, (2) S-locus mRNAs accumulate in a precise organ-specific pattern during floral development, and (3) the ability to inhibit the growth of incompatible pollen tubes appears to require a threshold accumulation of the stylar gene product, along with the participation of as yet undefined pollen gene products.

INTRODUCTION

Self-incompatibility, a genetic barrier to inbreeding found in almost half the families of flowering plants, is thought to have played a major role in the success and evolution of angiosperms (see de Nettancourt, 1977; Nasrallah and Nasrallah, 1986; Bernatzky et al., 1988, for reviews of self-incompatibility). In most systems studied to date, the expression of self-incompatibility is governed by a single, multiallelic locus, known as the S-locus (de Nettancourt, 1977; Tanksley and Loaiza-Figueroa, 1985). In gametophytic self-incompatibility, pollen tubes expressing S-alleles identical to those expressed in the style cease growth in the upper third of the style, whereas pollen tubes lacking S-alleles in common with the style grow normally and effect fertilization. Although S-locus genes have recently been cloned from both gametophytic and sporophytic self-incompatible species (Anderson et al., 1986, 1989; Nasrallah et al., 1985, 1988; Kirch et al., 1989), neither the molecular basis of S-allele recognition nor the nature of developmental programming governing S-locus expression in pollen and styles is understood.

One approach to studying self-incompatibility is to compare the structure and expression of S-alleles in self-incompatible varieties with that in related pseudo-self-compatible varieties showing qualitative or quantitative defects in the self-incompatibility response. Pseudo-self-compatibility has been studied extensively in *Petunia hybrida*, (Ascher, 1984), and two classes of pseudo-self-compatible lines have been defined. Pollen-part and stylar-part mutant lines show a unilateral, organ-specific breakdown in the expression of self-incompatibility (Flaschenriem and Ascher, 1979a, 1979b). A second type of pseudo-self-compatibility is characterized by the quantitative breakdown of self-incompatibility. In this case, variable levels of seed set are observed following crosses that would normally be expected to be incompatible and produce no seed (Ascher, 1984).

In this study, we isolated and characterized three different S-alleles of *P. hybrida* and compared the expression of those alleles in self-incompatible and pseudo-self-compatible varieties. We show that the temporal and spatial regulation of S-allele expression is tightly linked to the acquisition of self-incompatibility. The ability to inhibit incompatible pollen tubes appears to require a threshold level of the stylar gene product. Comparison of the derived amino acid sequences for these alleles shows that two of the alleles share over 90% protein sequence similarity. Because the sequence variability that does exist is clustered in two regions of the protein, we propose that limited sequence differences between stylar S-alleles may confer recognition specificity. Finally, because stylar S-locus mRNA sequences were undetectable in pollen and appeared to be expressed normally in a pseudo-self-compat-

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ible line, we conclude that the pollen-expressed component of self-incompatibility may be distinct from that expressed in styles.

RESULTS

Isolation of Cloned Stylar mRNAs Encoding the S_1, S_2, and S_3 Alleles of the Petunia Self-Incompatibility Locus

To isolate S-alleles from *P. hybrida*, we obtained lines having defined S_1, S_2, and S_3 alleles (Flaschenriem and Ascher, 1979a, 1979b), and constructed stylar mRNA libraries in λZAP from the $S_{1.2}$ and $S_{3.3}$ varieties. We screened these libraries by differential plaque hybridization to obtain style-specific clones, and in addition screened the libraries directly with an oligonucleotide homologous to an mRNA encoding a region of 15 amino acids found to be highly conserved among S-associated proteins of *Nicotiana alata* and *Lycopersicon peruvianum* (Mau et al., 1986). These screens resulted in isolation of the cDNA clones designated PS1B (S_1 allele, $S_{1.2}$ library), PS2A (S_2 allele, $S_{1.2}$ library), and PS3A (S_3 allele, $S_{3.3}$ library), as well as several style-specific clones unrelated to the S-locus.

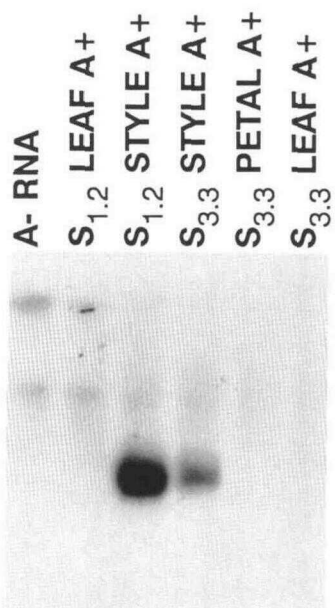


Figure 1. Hybridization of an S-Allele Oligonucleotide to *P. hybrida* mRNA.

Polyadenylated RNA (2 μg from each organ as indicated) or poly(A^-) RNA (10 μg from $S_{1.2}$ leaves) was electrophoresed on 1% agarose-formaldehyde gels, blotted to a nylon membrane, and hybridized with an oligonucleotide homologous to an mRNA encoding the conserved N-terminal sequence of S-allele proteins of *N. alata* and *L. peruvianum* (see Methods). Hybridization was under conditions of relatively low stringency (50% formamide, 1 M Na^+, 24°C). The weak background bands are due to nonspecific hybridization with residual ribosomal RNA.

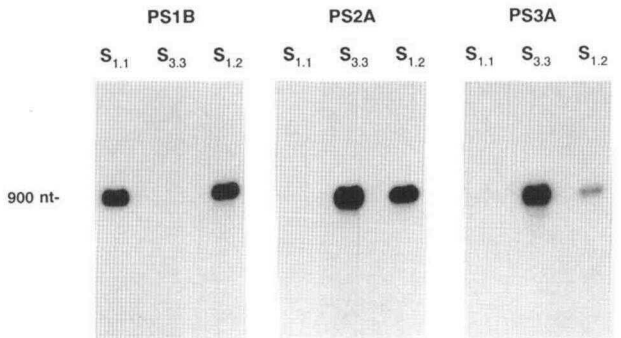


Figure 2. Hybridization of cDNA Clones to Stylar mRNA.

Polyadenylated RNA (1 μg) from mature styles of $S_{1.1}$, $S_{1.2}$, or $S_{3.3}$ varieties was electrophoresed on 1% agarose-formaldehyde gels and blotted to nylon membranes. Membranes were hybridized to each of the individual cDNA clones under conditions of moderate stringency (50% formamide, 1M Na^+, 42°C).

Figure 1 shows that the oligonucleotide hybridized to a 900 nt mRNA found in styles of $S_{1.2}$ and $S_{3.3}$ petunia, but did not hybridize detectably to mRNAs from leaves or petals of these same lines. Figure 2 shows that all three cDNA clones hybridized to an mRNA of approximately 900 nt that accumulated to high levels in styles of $S_{1.1}$, $S_{1.2}$, and $S_{3.3}$ petunia. PS1B hybridized strongly to mRNA from $S_{1.1}$ and $S_{1.2}$ styles, but weakly to mRNA from $S_{3.3}$ styles. PS2A and PS3A cDNAs hybridized strongly to mRNA from $S_{1.2}$ and $S_{3.3}$ styles, but only weakly to mRNA from $S_{1.1}$ styles. These results indicate that the $S_{1.2}$ style mRNA having sequence similarity to the PS1B cDNA is the S_1 mRNA, whereas the mRNA having sequence similarity to the PS2A cDNA is the S_2 mRNA.

Structure of S-Allele Genomic Sequences

To investigate the nature of genomic sequences encoding the putative S-alleles, we used the cDNA clones to screen libraries of $S_{1.1}$, $S_{1.2}$, and $S_{3.3}$ genomic DNAs. Figure 3 shows maps of the cDNA clones along with partial maps of the chromosomal regions in $S_{1.1}$, $S_{1.2}$, and $S_{3.3}$ genomic DNA encoding these genes. These maps show that a large degree of restriction site polymorphism is present among these three alleles.

To estimate the gene copy number, we hybridized digests of petunia genomic DNA with the individual cDNA clones. Figure 4 shows that each cDNA clone hybridized prominently with a single genomic fragment from each petunia genotype. The PS1B cDNA hybridized to a 4.8-kb EcoRI fragment found in the $S_{1.1}$ and $S_{1.2}$ lines, the PS2A cDNA hybridized to a different (2.3-kb EcoRI) fragment in

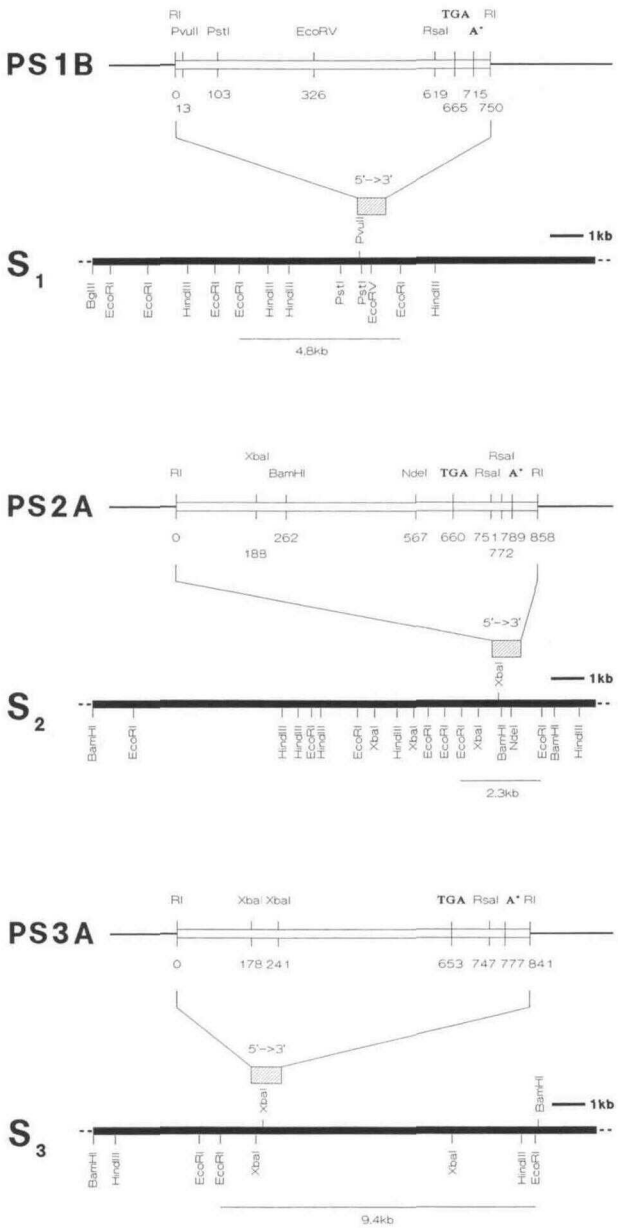


Figure 3. Restriction Maps of Petunia S-Alleles.

Restriction maps for the cDNA clones PS1B (S_1 allele), PS2A (S_2 allele), and PS3A (S_3 allele) are shown together with maps of the genomic regions in the vicinity of these genes. Sites on the cDNA clones labeled RI are from the EcoRI/SmaI adapters used for cDNA cloning (see Methods) and do not represent sites in the petunia genome. The hatched box shows the location of homology of the cDNA clones to genomic DNA. EcoRI fragments shown in Figure 4 are depicted as lines below the genomic DNA maps. Sites labeled TGA and A^+ in the cDNAs indicate the locations of the stop codons and polyadenylation signals, respectively.

the $S_{1.2}$ strain but did not hybridize to the $S_{1.1}$ DNA, and the PS3A cDNA hybridized to a 9.4-kb EcoRI fragment from the $S_{3.3}$ strain (see map in Figure 3). As expected from the RNA hybridization data, the PS2A and PS3A cDNAs show cross-reactivity, with the PS2A clone detecting the 9.4-kb EcoRI S_3 band and the PS3A clone detecting the 2.3-kb EcoRI S_2 band. We detected similar restriction fragment length polymorphisms among the three alleles when we used enzymes other than EcoRI (see the genomic DNA maps in Figure 3). We did not observe polymorphic restriction fragments when we hybridized DNA blots with cloned DNAs not related to the S-locus (three style-specific/non-S cDNAs, *rbcS*, and *Cab*). In each case, EcoRI and HindIII restriction fragments of identical size were observed in all three backgrounds (J. Okuley and R. Clark, data not shown). Together with the RNA hybridization data, these findings indicate that (1) the PS1B, PS2A, and PS3A cDNAs represent the S_1, S_2, and S_3 alleles, respectively; (2) the S_2 and S_3 alleles share extensive sequence similarity, while both are diverged from the S_1 allele; and (3) the pattern of hybridization seen to DNAs of different S-genotypes is that expected for different alleles of a locus.

DNA Sequence Analysis of the Petunia S-Alleles

To more completely characterize the structure and rela-

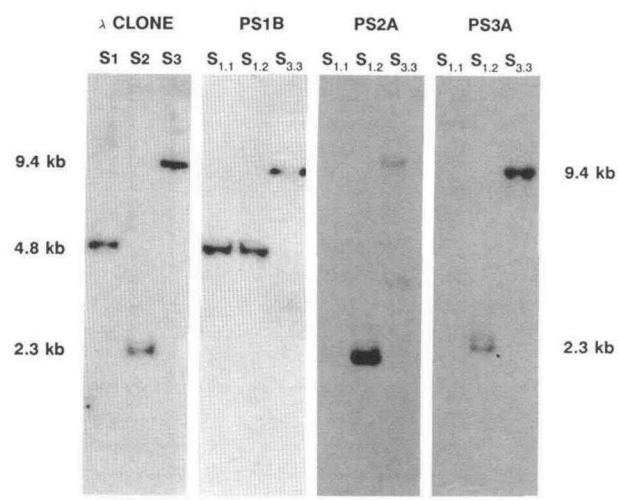


Figure 4. Organization of S-Locus Genomic Sequences.

DNA from genomic clones in λJ1 (S1, S2, S3) or petunia genomic DNA ($S_{1.1}$, $S_{1.2}$, $S_{3.3}$) was digested with EcoRI and analyzed by blot hybridization with the PS1B, PS2A, and PS3A cDNA clones. PS1B: Hybridization with the PS1B cDNA. PS2A: Hybridization with the PS2A cDNA. PS3A: Hybridization with the PS3A cDNA. The identity of the strongly hybridizing bands is described in the text. The weakly hybridizing 2.6-kb band in $S_{1.2}$ DNA has not yet been identified. The signal in the $S_{3.3}$ lane hybridized with the PS1B cDNA is due to nonspecific background contamination.

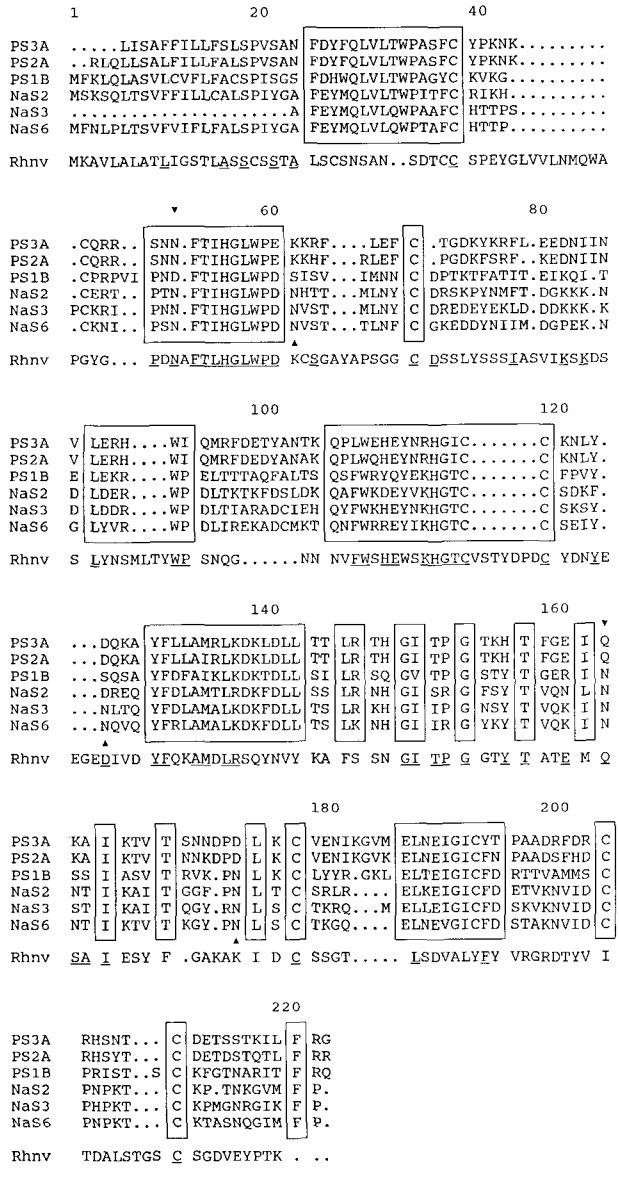


Figure 5. Derived Amino Acid Sequences of S-Allele Proteins.

DNA sequences for each of the petunia cDNA clones (PS3A, PS2A, PS1B) were translated to give derived amino acid sequences for the putative S-allele proteins. The derived amino acid sequences were aligned with those for the *N. alata* alleles (NaS2, NaS3, NaS6) (Anderson et al., 1986, 1989) and the Rh ribonuclease of *R. niveus* (*Rhnv*) (Horiuchi et al., 1988) using GAP (Devereux et al., 1984) with a gap weight of 3.0 and a length weight of 0.1. Boxed sequences show regions of greater than 80% average residue similarity based on the scoring table of Gribskov and Burgess (1986). *R. niveus* residues showing identity with S-allele residues are underlined. Consensus sequences for glycosylation (NXT/NXS) are shown by ▼ for the petunia alleles and by ▲ for the *N. alata* alleles. Sequence numbers shown refer to the PS1B protein sequence.

tionships among the three putative S-alleles, we determined the DNA sequence for each of the cloned cDNAs. Figure 5 shows the derived amino acid sequences for the three alleles from *P. hybrida* aligned with amino acid sequences for S-alleles isolated from *N. alata* (Anderson et al., 1986, 1989). Several regions of high protein sequence conservation (greater than 80% average residue similarity) are shown boxed in Figure 5. Several individual residues are also conserved, including nine cysteine residues. Three regions, depicted as open boxes in Figure 6, show high variability (less than 40% average residue similarity) in amino acid sequence among all six alleles. Using the conserved petunia sequence regions to search the Genbank and EMBL Databases, we found blocks of sequence homology (shown underlined in Figure 5) between the petunia proteins and a ribonuclease from *Rhizopus niveus* (Horiuchi et al., 1988; see also McClure et al., 1989).

To investigate whether regions showing either a high degree of sequence conservation or sequence variability were more likely to be buried in the interior of the protein or found on the surface, we aligned the homology profiles of Figure 5 with hydropathy plots for the S_1, S_2, and S_3 alleles. Figure 6A shows the profile for the S_1 allele, while the S_2 and S_3 profiles are shown superimposed in Figure 6B. Of the highly conserved regions, the most N-terminal (S_1 residues 24 to 38, S_2 residues 22 to 36, S_3 residues 19 to 33) and the most C-terminal (S_1 residues 185 to 194, S_2 residues 183 to 192, S_3 residues 181 to 190) are quite hydrophobic in character. One conserved region (S_1 residues 105 to 119, S_2 residues 101 to 115, S_3 residues 99 to 113) is highly hydrophilic in all three alleles, and in addition shows strong homology to the active site region of the *R. niveus* ribonuclease (Horiuchi et al., 1988). These data indicate that the PS1B, PS2A, and PS3A cDNAs represent cloned mRNAs of the *P. hybrida* S-locus, that these alleles are homologous to those isolated from *N. alata* (Anderson et al., 1989), and that the petunia S-proteins share putative functional domains with known ribonucleases.

Spatial and Temporal Patterns of S-Allele Gene Expression

To determine whether the accumulation of mRNA homologous to the cloned cDNAs is organ specific, we isolated RNA from styles, leaves, petals, ovaries, anthers, and pollen germinated in vitro, and hybridized a slot blot of the different RNAs to the labeled cDNAs. As can be seen in Figure 7A, all three cDNA clones hybridized strongly and specifically to mRNA from styles. No detectable hybridization of any of the cDNAs was observed to mRNA from leaves, petals, anthers, or germinated pollen. Long exposure of sensitive RNA gel blots, however, as shown in Figure 7B, demonstrated a low level of hybridization with mRNA isolated from ovarian tissue. As a further test of the potential expression of style-expressed sequences in

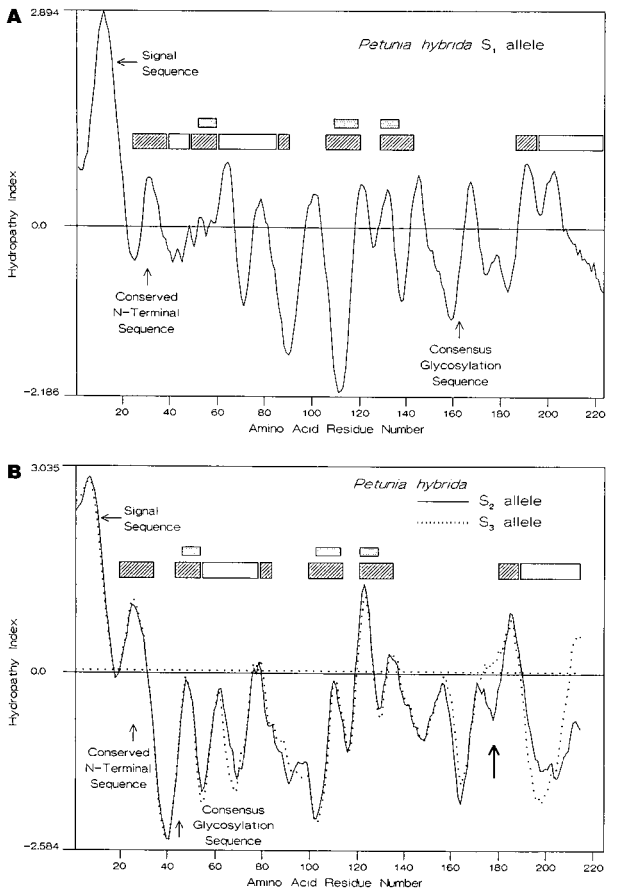


Figure 6. Hydropathy Profiles of S-Allele Proteins.

(A) S_1 protein. The hydropathy profile (Kyte and Doolittle, 1982) for the S_1 protein was derived using a window of seven residues and one cycle of smoothing. Boxes above the hydropathy profile show the location of regions of high sequence conservation or variability among the S-allele proteins shown in Figure 5. Cross-hatched boxes denote regions showing greater than 80% average residue similarity among all six alleles; open boxes show variable regions of less than 40% average residue similarity (Gribskov and Burgess, 1986). Stippled boxes show the location of *R. niveus* ribonuclease Rh sequences showing strong homology with the S-allele sequences.

(B) S_2 protein. The hydropathy profile (Kyte and Doolittle, 1982) of the S_2 protein was derived using a window of seven residues and one cycle of smoothing. Cross-hatched boxes denote regions showing greater than 80% average residue similarity among all six alleles. The open boxes show regions having the greatest amount of sequence variability between the S_2 and S_3 alleles (see text). Stippled boxes show regions of *R. niveus* ribonuclease Rh sequence showing strong homology with the S-allele sequences. Large arrow indicates area of major difference between the hydropathy plots of the S_2 and S_3 proteins.

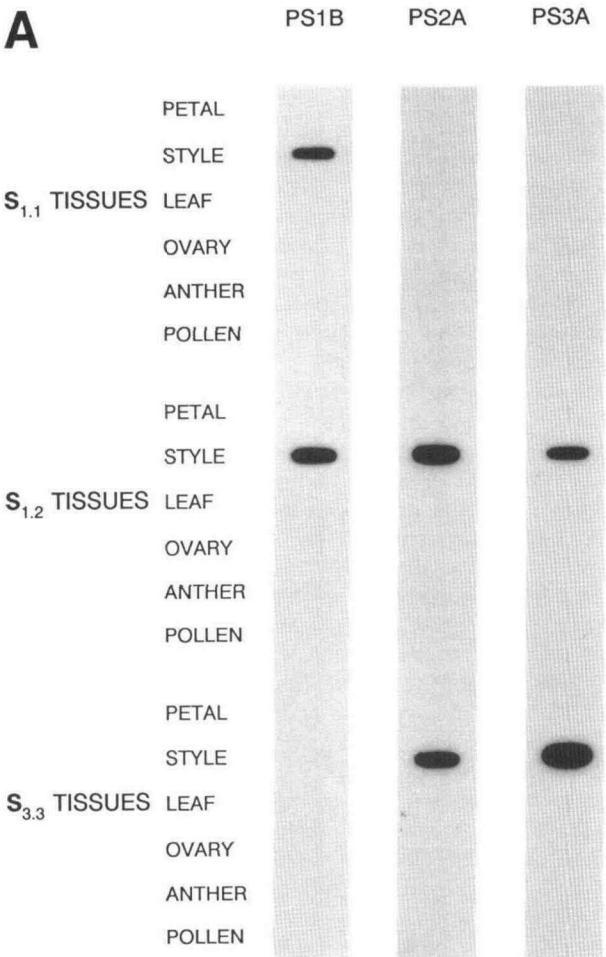
pollen, high-sensitivity RNA blots containing 10 μg of poly(A^+) RNA isolated from mature anthers of the $S_{1.2}$ strain were hybridized with the PS1B cDNA clone. No hybridization was observed even after extended exposure (R. Clark, data not shown).

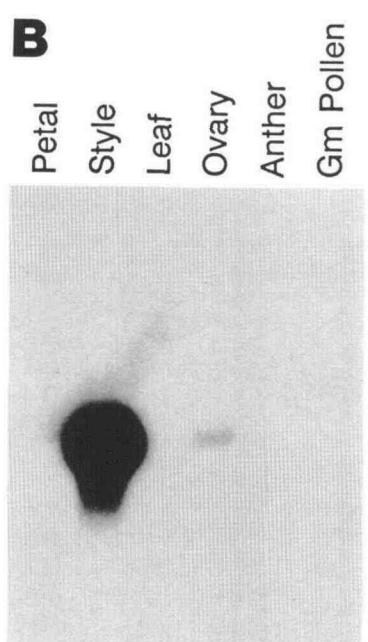
To investigate whether accumulation of S-locus mRNA paralleled the acquisition of self-incompatibility, we used a quantitative slot-blot assay to measure the relative level of PS1B mRNA at different stages of floral development. Figure 8A shows that PS1B mRNA accumulated to high levels over the course of floral development, with a large increase between −3 days and maturity. Bud pollination of flowers from the $S_{1.1}$ line at different stages of development demonstrated that mature flowers and floral buds 1 or 2 days prior to anthesis were capable of preventing fertilization by self-pollen, whereas pollination of floral buds 3 days prior to anthesis resulted in capsule formation and seed set (T. Sims and P.D. Collins, data not shown). Thus, the greatest accumulation of S_1 mRNA occurs during the period when the style undergoes the transition from self-compatibility to self-incompatibility.

To compare the accumulation of different S-alleles in a heterozygous line, we hybridized either the PS1B or PS2A cDNAs to slot blots of total RNA isolated from $S_{1.2}$ styles at different developmental stages. Figure 8B shows that although the temporal pattern of expression was similar for the S_1 and S_2 genes, the accumulation of the S_2 mRNA was substantially greater than that of the S_1 mRNA in the same tissue. At stage −3, during which bud pollination of the $S_{1.1}$ plant resulted in seed set, the level of S_2 mRNA in the $S_{1.2}$ plant was about eightfold greater than the level of S_1 mRNA. In a separate experiment, we had bud pollinated the $S_{1.2}$ stock at stage −3 to produce seed. When we analyzed the F1 progeny by DNA blot analysis, we found only $S_{1.1}$ or $S_{1.2}$ plants with no $S_{2.2}$ plants observed ($\chi^2 = 7.82$, $P < 0.03$) (J. Okuley, unpublished observations). Taken together, these data demonstrate that (1) S-locus mRNAs accumulate in a precise organ-specific pattern in reproductive tissue, (2) there is a direct correlation between the accumulation of S-allele mRNA sequences and the ability to reject self pollen tubes, and (3) the level of S_2 mRNA at stage −3 is apparently sufficient to render the $S_{1.2}$ plants incompatible to S_2 pollen tubes.

Sequences Homologous to the S_1 Allele Are Expressed in a Pseudo-Self-Compatible Petunia Strain

One form of pseudo-self-compatibility is characterized by variable levels of seed set following crosses that would normally be expected to be incompatible and produce no seed (Ascher, 1984). This type of pseudo-self-compatibility appears to be present in *P. hybrida* MSU1093, a variety that gives consistently high levels of seed set on selfing of mature flowers (T. Sims, unpublished data). Because the





levels of S-allele mRNA sequences in immature floral buds of the self-incompatible varieties appeared to be too low to allow inhibition of incompatible pollen tubes (see Figure 8), we wished to determine whether pseudo-self-compatibility in MSU1093 resulted from decreased expression of the S-locus.

Figure 9A demonstrates that the PS1B cDNA hybridized strongly to a HindIII restriction fragment in MSU1093 DNA that was the same size (4.2 kb, see also the genomic maps in Figure 3) as that in the $S_{1.1}$ and $S_{1.2}$ lines. Weak hybridization was also seen to a larger (6 kb) fragment in MSU1093 that was not seen to hybridize in the incompatible lines. RNA gel blot hybridization demonstrated the presence of a stylar mRNA in MSU1093 that was identical in size to that from the $S_{1.1}$ line (R. Clark, data not shown). To determine whether S-locus expression was decreased in MSU1093 relative to the self-incompatible variety, we used a quantitative slot blot assay to compare the levels of S-locus mRNA in mature styles of MSU1093 with that in $S_{1.1}$ styles. As shown in Figure 9B, we found that the level of hybridizing mRNA was identical in the two varieties.

S-alleles from both *P. hybrida* and *N. alata* show regions of sequence homology with active sites of fungal ribonucleases (see Figure 5). In addition, direct assays using purified S-allele proteins (McClure et al., 1989) indicate that these proteins have ribonuclease activity. These findings suggest that ribonuclease activity associated with S-locus encoded proteins may be the causal agent of pollen tube inhibition. When we compared the level of ribonuclease activity in crude extracts of $S_{1.1}$ and MSU1093 styles, however, we found equal levels of activity in self-incompatible and pseudo-self-compatible styles (31 A_{260} min^{-1} mg^{-1} for $S_{1.1}$ styles, 33 A_{260} min^{-1} mg^{-1} for MSU1093 styles). Together, these findings show that the S-locus is intact in MSU1093 and that at the level of mRNA stylar S-allele expression is identical to that in self-incompatible lines. The defect in self-incompatibility in MSU1093 appears, therefore, to result from some factor other than decreased expression of the stylar S-allele.

Figure 7. Hybridization of cDNA Clones to mRNAs Isolated from Different Organs.

(A) Total RNA (10 μg) from leaves, petals, styles, ovaries, mature anthers, and pollen germinated in vitro, isolated from $S_{1.1}$, $S_{1.2}$, and $S_{3.3}$ plants, was applied to nylon membranes using a slot blotter apparatus, and hybridized with each of the labeled cDNA clones.

(B) Poly(A^+) RNA (2 μg) was isolated from different organs (petals, styles, leaves, ovaries, anthers, or pollen germinated in vitro) of the $S_{1.2}$ strain, electrophoresed on a formaldehyde agarose gel, and blotted to a nylon membrane. The RNA was hybridized to the PS2A cDNA probe under conditions of moderate stringency (50% formamide, 1 M Na^+, 42°C).

DISCUSSION

The PS1B, PS2A, and PS3A cDNA Clones Represent Stylar-Expressed mRNAs Encoded by the Petunia S-Locus

One of the major conclusions from our data is that self-incompatibility requires the precise developmental regulation of complex interacting components. The mechanism of recognition between pollen tubes and styles in incompatible species is unknown, although it is clear that one component of this recognition resides in stylar expressed proteins encoded by the S-locus. We have isolated cloned mRNAs that accumulate to high levels in mature styles, are present at much lower levels in ovaries, and are undetectable in leaves, petals, mature anthers, or pollen germinated in vitro. These genes are expressed at low levels early in floral development and show a dramatic increase in expression that is correlated in time with the transition from self-compatibility to self-incompatibility.

DNA blot hybridization experiments indicated that DNA sequences homologous to the cDNA clones were of low copy number in the petunia genome and were associated with polymorphic restriction fragments that hybridized in a manner expected for allelic sequences. That is, single, distinct, restriction fragments from the $S_{1.1}$ and $S_{3.3}$ homozygous lines hybridized to the cDNAs, whereas two fragments hybridized in the $S_{1.2}$ heterozygote. One of the $S_{1.2}$ bands (from the S_1 allele) was identical in size to that which hybridized in the $S_{1.1}$ strain with the other (S_2) fragment of a different size. Separate, nonallelic genes did not show this pattern of hybridization but hybridized to identical restriction fragments in each of our petunia lines. Taken together, these data indicate that the PS1B, PS2A, and PS3A clones represent stylar-expressed alleles associated with the self-incompatibility locus of *P. hybrida*.

The Petunia S-alleles Show Structural and Sequence Similarity to Other S-Locus-Associated Proteins

DNA sequence analysis showed that the proteins encoded by the S_1, S_2, and S_3 mRNAs have isoelectric points and N-terminal amino acid sequences similar to those of putative S-allele proteins from *P. hybrida* identified by gel electrophoresis (Kamboj and Jackson, 1986; Broothaerts et al., 1989). Predicted mol wt and isoelectric points are: 22,800/9.6 for the S_1 protein, 23,600/9.2 for the S_2 protein, and 23,700/9.3 for the S_3 protein. The mol wt predicted from the derived amino acid sequence are 15% to 25% less than those estimated from gel electrophoresis. This difference is most likely attributable to the effect of carbohydrate chains on the migration of the proteins in SDS gels. The petunia S-alleles have signal peptides characteristic of secreted proteins (von Heijne, 1986). Cleavage rules for secreted proteins (von Heijne, 1986) predict that the mature S_1 protein should begin with serine (S_1 residue no. 23) and both the S_2 and S_3 proteins with asparagine (S_2 residue no. 21, S_3 residue no. 18).

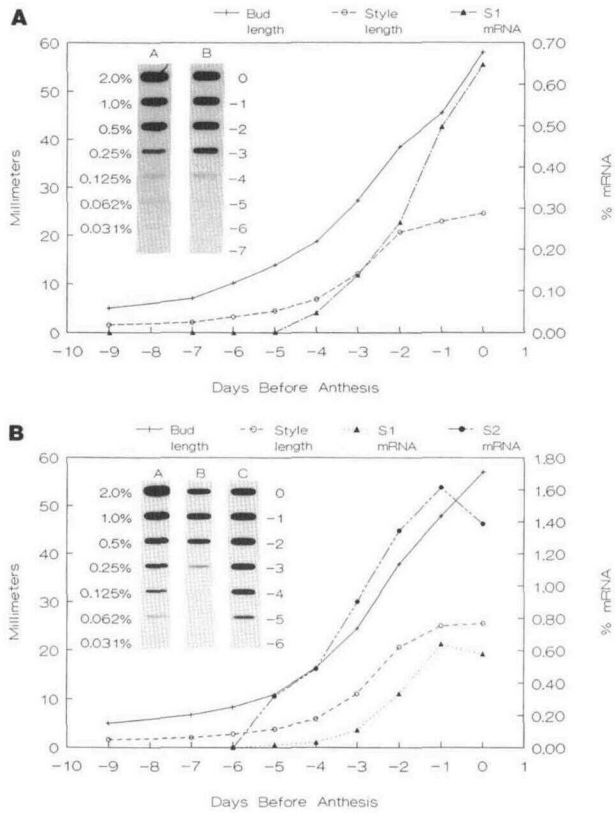


Figure 8. Developmental Expression of S-Locus Genes.

(A) $S_{1.1}$ strain. Total RNA isolated from styles of staged floral buds, or RNA transcribed in vitro from the PS1B cDNA clone, was applied to a nylon membrane using a slot blotter apparatus and hybridized with the PS1B cDNA. Following autoradiography, individual slot-bands were cut out and counted in a liquid scintillation counter. Slot blot: Lane A, reconstruction with RNA transcribed in vitro from the PS1B cDNA clone. Transcribed RNA was serially diluted and applied to membrane slots in amounts corresponding to 2.0%, 1.0%, 0.5%, 0.25%, 0.125%, 0.062%, and 0.031% of the mRNA mass, assuming that mRNA comprised 1.0% of the total RNA. Lane B, total RNA (1 μg load shown) isolated from styles of staged floral buds. Numbers indicate days prior to anthesis (bud stage −9 not shown). Graph: (+) floral bud length, (○) style length, (▲) estimated levels of S_1 mRNA (as percent of mRNA mass) at different stages of development.
(B) $S_{1.2}$ strain. RNA levels were determined as described for **(A)**. Slot blot: Lane A, reconstruction for S_2 mRNA (S_1 RNA reconstruction not shown). Lane B, total RNA (1 μg load shown) from styles of staged floral buds hybridized to the PS1B probe. Lane C, total RNA (1 μg load shown) from styles of staged floral buds hybridized to the PS2A probe. Graph: (+) bud length, (○) style length, (▲) S_1 mRNA, (●) S_2 mRNA.

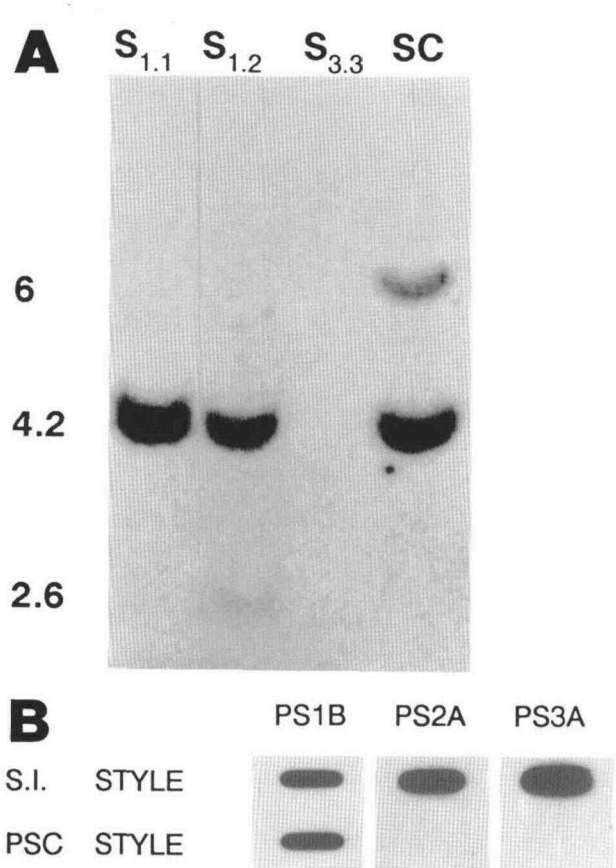


Figure 9. Homologous Sequences in Pseudo-Self-Compatible *P. hybrida*.

(A) DNA blot hybridization. Genomic DNA (10 μg) from $S_{1.1}$, $S_{1.2}$, $S_{3.3}$, or MSU1093 (SC) plants was digested with *HindIII* and analyzed by hybridization with the PS1B cDNA clone.
(B) RNA slot hybridization. Total RNA (10 μg) from mature styles of $S_{1.1}$, $S_{1.2}$, $S_{3.3}$, and MSU1093 was applied to membranes using a slot blotter followed by hybridization with the individual cDNA clones. S.I. style: RNA from $S_{1.1}$ (PS1B), $S_{1.2}$ (PS2A), or $S_{3.3}$ (PS3A) styles. PSC style: RNA from MSU1093 styles. No hybridization to RNA from MSU1093 styles is seen with either the S_2 or S_3 cDNAs.

Analysis of derived peptide sequences of the petunia S-alleles showed several regions that are highly conserved in primary amino acid sequence separated by regions of sequence variability. These conserved regions are homologous to those seen in S-alleles of *N. alata* (see Figure 5) (Anderson et al., 1989). One conserved region (S_1 residues 105 to 119) containing sequences homologous to those thought to form the active site of fungal ribonucleases is extremely hydrophilic and is probably exposed to the solvent. Six of the nine cysteine residues conserved among the different S-alleles are found in analogous positions in RNase Rh from *R. niveus*. Because the RNase Rh cysteines and those in the related RNase T_2 from *Aspergillus oryzae* are all components of disulfide linkages (Horiuchi et al., 1988; Kawata et al., 1988), it seems probable that these residues contribute to stabilization of tertiary structure in the S-alleles.

Sequence Comparison of the S_2 and S_3 Alleles Suggests Limited Amino Acid Changes May Result in Altered Recognition Specificity

The high degree of sequence conservation exhibited between the S_2 and S_3 proteins suggests that very limited sequence differences may confer allelic specificity. Only 18 positions between the S_2 and S_3 proteins show nonconservative amino acid changes, and the changes that do exist appear to be clustered. Thirteen of the 18 replacements (72%) occur in the two variable regions shown as open boxes in Figure 6B. Unlike the *N. alata* alleles (Anderson et al., 1989), much of the sequence variability occurs at the carboxy terminus of the protein. Also, the only significant differences in the hydropathy profiles of S_2 and S_3 occur in regions near the carboxy terminus of the protein. The more hydrophilic profile of the S_2 protein in this region results from the presence of lysine 183 in S_2 versus methionine 178 in S_3 (see large arrow in Figure 6B) and from an isoleucine residue (position 217) at the C terminus of the S_3 protein.

All S-alleles characterized to date are glycoproteins (Bernatzky et al., 1988), and experiments using tunicamycin (Sarker et al., 1988) suggest that glycosylation is required for S-protein function. Each of the three petunia alleles has a single consensus sequence (NXT/NXS) for glycosylation. This sequence occurs at the same position in the S_2 and S_3 proteins and is located near the N terminus (Figure 6B). By contrast, the single site in the S_1 protein is located near the carboxy terminus (Figure 6A). As the single glycosylation signal in S_2 and S_3 occurs at the same position in the two proteins, it seems unlikely that glycosylation alone can determine specificity. Woodward et al. (1989), however, have noted that the *N. alata* S-glycoproteins show variation in the number, type, and fine structure of glycan chains, even though the positions of four glycosylation sites are conserved among three different S-alleles. We have not characterized the degree of glycosylation of the petunia proteins so we do not know whether this site is in fact glycosylated in both proteins, or whether the number or composition of glycan chains is different between the S_2 and S_3 alleles.

Developmental Expression of S-Alleles in Self-Incompatible Petunia

The petunia S-allele mRNAs showed a distinct temporal pattern of accumulation that was correlated in time with the transition from bud self-compatibility to self-incompatibility. When we analyzed the levels of S-allele mRNA in

the $S_{1.2}$ heterozygote, however, we found that the S_2 mRNA accumulated to a level several-fold higher than the S_1 mRNA. Bud pollination of the $S_{1.2}$ heterozygote failed to produce any $S_{2.2}$ plants. These results suggest that the level of S_2 protein in the $S_{1.2}$ style at the stage used for bud pollination was sufficient to inhibit S_2 pollen, but that the S_1 protein had not accumulated to a level that allowed inhibition of the S_1 pollen. At this same bud stage, the level of S_1 mRNA in $S_{1.2}$ styles was identical to that in $S_{1.1}$ styles—where bud pollination resulted in a high level of seed set. These data suggest that the ability to inhibit self pollen is a quantitative threshold effect, in which a sufficient amount of S-encoded protein must accumulate in order to inhibit pollen tube growth.

S-Allele Sequences Are Highly Expressed in a Pseudo-Self-Compatible Petunia Variety

DNA and RNA blot hybridizations showed that genomic sequences homologous to the S_1 allele were present in the pseudo-self-compatible strain MSU1093 and were expressed in mature styles at a level indistinguishable from that in the self-incompatible plant. We found no correlation between the overall level of ribonuclease activity in crude style extracts and the presence of self-incompatibility. The level of ribonuclease activity in the pseudo-self-compatible MSU1093 variety was identical to that found in the self-incompatible $S_{1.1}$ line. Preliminary assays indicate that the level of ribonuclease activity in the $S_{1.2}$ strain is also similar to the levels observed in $S_{1.1}$ and MSU1093 styles, even though S-allele mRNA accumulates to greater prevalence in $S_{1.2}$ styles. These data contrast somewhat with those reported by McClure et al. (1989), where substantial differences in ribonuclease levels were observed between self-incompatible *N. alata* and self-compatible *N. tabacum*. The level of ribonuclease activity in petunia styles (30 A_{260} min^{-1} mg^{-1}) was lower than that reported by McClure et al. (1989) for *N. alata* (180 to 2200 A_{260} min^{-1} mg^{-1}), but is approximately equivalent to that found in self-incompatible *Lycopersicon peruvianum* (26 A_{260} min^{-1} mg^{-1} (I. Lewis, B. McClure, and A. Clarke, personal communication). The level of ribonuclease activity in a self-compatible accession of *L. peruvianum* is approximately 10-fold below that of the self-incompatible plant (I. Lewis, B. McClure, and A. Clarke, personal communication). Taken together, these data indicate that the defect in self-incompatibility in MSU1093 may result from the inability of stylar proteins to recognize pollen tubes or from inactivation of self-incompatibility in the pollen. Although the large amount of sequence conservation between fungal ribonucleases and the S-alleles of different Solanaceous species suggests that ribonuclease activity may be involved in pollen tube inhibition, it is not yet clear whether self-incompatibility in *P. hybrida* is correlated with stylar ribonuclease activity.

What Are the Different Components Required for the Self-Incompatibility Response?

Previous models for the mechanism of self-incompatibility postulated that style-expressed and pollen-expressed components were encoded by the same genetic locus (de Nettancourt, 1977) with dimers formed between identical S-allele products from pollen and styles. Our data suggest that S-locus sequences expressed in pollen and styles are products of different genes. We have been unable to detect expression of sequences having homology with the stylar-expressed genes in either mature anthers or germinated pollen, even on high-sensitivity RNA blots which are able to detect sequences present at 10^{-4}% of the mRNA (Fischer and Goldberg, 1982). In addition, we have used transient expression assays to demonstrate that 5′ flanking sequences of the S_1 allele can direct specific stylar expression of the β-glucuronidase reporter gene. To date, we have failed to observe any expression in mature pollen using these same constructs, whereas a CaMV-35S/GUS construct gave high levels of pollen expression in these same assays (R. Clark, unpublished data). The stylar component appears to be expressed normally in the pseudo-self-compatible line MSU1093. Although we cannot yet rule out the possibility that an altered protein sequence eliminates the ability of MSU1093 to bind pollen tubes, there appears to be no large alterations in the mRNA encoded by this gene. Also, transformation of *N. tabacum* with the stigma-expressed component from a sporophytic self-incompatible plant *Brassica oleracea* failed to result in self-incompatibility, even though the transferred gene was expressed to high levels in transmitting tissue of the style (Moore and Nasrallah, 1990).

The cloned S_1, S_2, and S_3 alleles appear, therefore, to represent a style-specific component of the *P. hybrida* self-incompatibility response. Proteins encoded by these genes possess two apparent functions: discrimination between pollen tubes of different allelic specificity and inhibition of incompatible pollen tubes. To test this hypothesis, we have transformed the $S_{3.3}$ variety with a genomic fragment containing the S_1 allele (J. Okuley, unpublished data). If pollen and style components are in fact encoded by separate genes, pollination of the transgenic plants with S_1 pollen should not result in seed set, whereas seed set should occur when transgenic progeny, homozygous for the introduced S_1 allele, are used as pollinators on $S_{1.1}$ style parents.

Finally, our data demonstrate that the expression of genes encoding the different component functions of self-incompatibility is highly regulated during floral development. Using transient expression assays, we have observed expression of S_1-β-glucuronidase (Jefferson, 1987) fusion genes in floral tissues where the stylar S-allele mRNA does not accumulate in vivo (R. Clark, manuscript in preparation). Because these tissues arise from a common cell lineage (Satina, 1944), they should possess in

common binding factors capable of activating lineage-specific gene expression (Davidson, 1989). According to this model, negative spatial control factors would be present in floral tissues in which the S-locus is silent (Davidson, 1989). What these factors are, the S-locus sequences with which they interact, and the mechanism by which self-incompatible plants regulate the developmentally specific expression of S-locus genes all remain to be determined.

METHODS

Genetic Stocks, Plant Growth, and Staging of Floral Development

Defined lines of *Petunia hybrida* having the genotypes $S_{1.1}$, $S_{1.2}$, and $S_{3.3}$ (Flaschenriem and Ascher, 1979a, 1979b) were obtained from Dr. Peter Ascher at the University of Minnesota. MSU1093 was obtained from Dr. Kenneth Sink of Michigan State University. Seeds were germinated on a commercial starter mix and transplanted to soil-less potting mix when about 2 weeks old. Plants were maintained either in growth chambers or in the greenhouse under 16-hr photoperiods and temperatures as close as possible to 20°C day/15°C night. Individual lines were tested for self-incompatibility by hand pollination and maintained by vegetative cuttings. For developmental staging of floral buds, individual buds were tagged as early as they could be identified as such (stage −9, bud length 5 mm), and the length of the bud measured at identical times on successive days until corolla opening and anthesis. A minimum of 20 measurements were made for each floral stage, standard deviations calculated, and cut-off values determined. Floral buds to be tested for self-incompatibility were emasculated, hand pollinated using a different flower from the same plant as pollen donor, tagged, and the flower covered by a glassine envelope to prevent contamination by stray pollen. All unused flowers were cut off daily to reduce stray pollen. Self-incompatibility was assayed by scoring for seed-capsule formation.

Oligonucleotide Hybridization Probe

An oligonucleotide (5′-AAAAGTGATTGGCCATGTTAACACGAGT-TGCATATACTCGAAAGC-3′) homologous to the *Nicotiana alata* S_2 allele (Anderson et al., 1986) was synthesized on an Applied Biosystems synthesizer and purified by HPLC chromatography.

RNA, DNA, and Protein Isolation, Blot Hybridization, and DNA Sequencing

Total RNA was isolated via a modification (Sims and Hague, 1981) of the method of Chirgwin et al. (1979), and poly(A^+) RNA was isolated by affinity chromatography over oligo(dT) cellulose. Genomic DNA was isolated as described by Fischer and Goldberg (1982). RNA gel electrophoresis and blotting followed protocols of Rave et al. (1979) and Thomas (1980). DNA sequencing of single-stranded DNA cloned into Bluescript (Stratagene) or pUC118/119 (Vieira and Messing, 1987) was performed as described previously (Nielsen et al., 1989). DNA sequence analysis used the programs of Devereux et al. (1984). Alignment of *Petunia* and *Nicotiana* sequences was accomplished using GAP, BESTFIT, and PRETTY of the UWGCG analysis package. Hydropathy plots employed the algorithms of Kyte and Doolittle (1982) with a window of 7 residues and one cycle of smoothing. Protein isolations and ribonuclease assays followed the protocols of McClure et al. (1989).

cDNA Library Construction and Screening

Poly(A^+) RNA from mature styles of $S_{1.2}$ and $S_{3.3}$ *P. hybrida* was used for synthesis of double-stranded DNA essentially according to the methods of Murray et al. (1983) and Gubler and Hoffman (1983). Double-stranded cDNA was made flush-ended with T4 DNA Polymerase (Gubler, 1987) and ligated to EcoRI/SmaI adapters (Boehringer-Mannheim). Excess adapters were removed by chromatography over Sepharose CL-4B (Eschenfeldt and Berger, 1987), and the cDNA ligated and packaged into λZAP (Stratagene Cloning Systems, 1988) according to the supplier's protocol. Libraries were screened by plaque hybridization (Benton and Davis, 1977) under conditions of reduced stringency (50% formamide, 1 M Na^+, 24°C). Positive plaques were purified and rescreened twice more, then converted to Bluescript SK^- plasmid clones (Stratagene) by plasmid rescue according to the supplier's protocol.

$S_{1.1}$, $S_{1.2}$, and $S_{3.3}$ Genomic Libraries and Clones

Random partial SAU3A genomic libraries of $S_{1.1}$, $S_{1.2}$, and $S_{3.3}$ *Petunia* were constructed in λJ1 (Loenen and Brammar, 1980) and screened by plaque hybridization (Benton and Davis, 1977) with the PS1B, PS2A, and PS3A cDNAs.

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REFERENCES

Anderson, M.A., McFadden, G.I., Bernatsky, R., Atkinson, A., Orpin, T., Dedman, H., Tregear, G., Fernley, R., and Clarke, A.E. (1989). Sequence variability of three alleles of the self-

incompatibility gene of *Nicotiana alata*. Plant Cell **1,** 483–491.

Anderson, M.A., Cornish, E.C., Mau, S., Williams, E.G., Hoggart, R., Atkinson, A., Bonig, I., Grego, B., Simpson, R., Roche, P.J., Haley, J.D., Penschow, J.D., Niall, H.D., Tregear, G.W., Coghlan, J.P., Crawford, R.J., and Clarke, A.E. (1986). Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. Nature **321,** 38–44.

Ascher, P.D. (1984). Self-Incompatibility. In Petunia: Monographs on Theoretical and Applied Genetics, Vol. 9, K.C. Sink, ed (Berlin: Springer-Verlag), pp. 92–109.

Benton, W.D., and Davis, R.W. (1977). Screening λgt recombinant clones by hybridization to single plaques in situ. Science **196,** 180–182.

Bernatzky, R., Anderson, M.A., and Clarke, A.E. (1988). Molecular genetics of self-incompatibility in flowering plants. Dev. Genet. **9,** 1–12.

Broothaerts, W.J., van Laere, A., Witters, R., Preaux, G., Decock, B., van Damme, J., and Vendrig, J.C. (1989). Purification and N-terminal sequencing of style glycoproteins associated with self-incompatibility in *Petunia hybrida*. Plant Mol. Biol. **14,** 93–102.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18,** 5294–5299.

Davidson, E. (1989). Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: A proposed mechanism. Development **105,** 421–445.

de Nettancourt, D. (1977). Incompatibility in Angiosperms. In Monographs on Theoretical and Applied Genetics, Vol. 3, R. Frankel, G.A.E. Gall, M. Grossman, and H.F. Linskens, eds (Berlin: Springer-Verlag).

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for VAX. Nucl. Acids Res. **12,** 387–395.

Eschenfeldt, W.H., and Berger, S.L. (1987). Purification of large double-stranded cDNA fragments. Methods Enzymol. **152,** 335–337.

Fischer, R.L. and Goldberg, R.B. (1982). Structure and flanking regions of soybean seed protein genes. Cell **29,** 651–660.

Flaschenriem, D.R. and Ascher, P.D. (1979a). Pollen tube expression of pseudo-self-compatibility (PSC) in *Petunia hybrida*. Theor. Appl. Genet. **54,** 97–101.

Flaschenriem, D.R. and Ascher, P.D. (1979b). S allele discrimination in styles of *Petunia hybrida* bearing stylar-conditioned pseudo-self-compatibility. Theor. Appl. Genet. **55,** 23–28.

Gribskov, M. and Burgess, R.R. (1986). Sigma factors from *E. coli*, *B. subtilis*, phage spo1, and phage T4 are homologous proteins. Nucl. Acids Res. **14,** 6745–6763.

Gubler, U. (1987). Second strand cDNA synthesis: mRNA fragments as primers. Methods Enzymol. **152,** 330–335.

Gubler, U., and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. Gene **25,** 263–269.

Horiuchi, H., Yanai, K., Takagi, M., Yano, K., Wakabayashi, E., Sanda, A., Mine, S., Ohgi, K., and Irie, M. (1988). Primary structure of a base non-specific ribonuclease from *Rhizopus niveus*. J. Biochem. **103,** 408–414.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Reporter **5,** 387–405.

Kamboj, R.K., and Jackson, J.F. (1986). Self-incompatibility alleles control a low molecular weight, basic protein in pistils of *Petunia hybrida*. Theor. Appl. Genet. **71,** 815–819.

Kawata, Y., Sakiyama, F., and Tamaoki, H. (1988). Amino-acid sequence of ribonuclease T_2 from *Aspergillus oryzae*. Eur. J. Biochem. **176,** 683–697.

Kirch, H.H., Uhrig, H., Lottspeich, F., Salamini, F., and Thompson, R.D. (1989). Characterization of proteins associated with self-incompatibility in *Solanum tuberosum*. Theor. Appl. Genet. **78,** 581–588.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157,** 105–132.

Loenen, W.A.M., and Brammar, W.J. (1980). A bacteriophage lambda vector for cloning large DNA fragments made with several restriction enzymes. Gene **20,** 249–259.

Mau, S-L., Williams, E.G., Atkinson, A., Anderson, M.A., Cornish, E.C., Grego, B., Simpson, R.J., Kheyr-Pour, A., and Clarke, A.E. (1986). Style proteins of a wild tomato (*Lycopersicon peruvianum*) associated with expression of self-incompatibility. Planta **169,** 184–191.

McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakiyama, F., and Clarke, A.E. (1989). Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. Nature **342,** 955–957.

Moore, H.M., and Nasrallah, J.B. (1990). A *Brassica* self-incompatibility gene is expressed in the stylar transmitting tissue of transgenic tobacco. Plant Cell **2,** 29–38.

Murray, M.G., Hoffman, L.M., and Jarvis, N.P. (1983). Improved yield of full-length phaseolin cDNA clones by controlling premature anticomplementary DNA synthesis. Plant Mol. Biol. **2,** 75–84.

Nasrallah, M.E., and Nasrallah, J.B. (1986). Molecular biology of self-incompatibility in plants. Trends Genet. **2,** 239–244.

Nasrallah, J.B., Kao, T.-H., Goldberg, M.L., and Nasrallah, M.E. (1985). A cDNA encoding an S-locus-specific glycoprotein from *Brassica oleracea*. Nature **318,** 263–267.

Nasrallah, J.B., Yu, S-M., and Nasrallah, M.E. (1988). Self-incompatibility genes of *Brassica oleracea*: Expression, isolation, and structure. Proc. Natl. Acad. Sci. USA **85,** 5551–5555.

Nielsen, N.C., Dickinson, C.D., Cho, T.-J., Thanh, V.H., Scallon, B.J., Fischer, R.L., Sims, T.L., Drews, G.N., and Goldberg, R.B. (1989). Characterization of the glycinin gene family in soybean. Plant Cell **1,** 313–328.

Rave, N., Crkvenjakov, R., and Boedtker, H. (1979). Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. Nucl. Acids. Res. **6,** 3559–3567.

Satina, S. (1944). Periclinal chimeras in *Datura* in relation to development and structure (a) of the style and stigma (b) of calyx and corolla. Am. J. Bot. **31,** 493–502.

Sims, T.L., and Hague, D.R. (1981). Light-stimulated increase in translatable mRNA for phosphoenolpyruvate carboxylase in leaves of maize. J. Biol. Chem. **256,** 8252–8255.

Stratagene Cloning Systems. (1988). Lambda ZAP instructional manual.

Tanksley, S.D., and Loaiza-Figueroa, F. (1985). Gametophytic

self-incompatibility is controlled by a single major locus on chromosome 1 in *Lycopersicon peruvianum*. Proc. Natl. Acad. Sci. USA **82,** 5093–5096.

Thomas, P.T. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77,** 5201–5205.

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. Methods Enzymol. **153,** 3–11.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucl. Acids Res. **14,** 4683–4690.

Woodward, J.R., Bacic, A., Jahnen, W., and Clarke, A.E. (1989). N-linked glycan chains on S-allele-associated glycoproteins from *Nicotiana alata*. Plant Cell **1,** 511–514.