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Hypervariable Domains of Self-Incompatibility RNases Mediate Allele-Specific Pollen Recognition

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Self-incompatibility (SI) in angiosperms is a genetic mechanism that promotes outcrossing through rejection of self-pollen. In the Solanaceae, SI is determined by a multiallelic *S* locus whose only known product is an S RNase. S RNases show a characteristic pattern of five conserved and two hypervariable regions. These are thought to be involved in the catalytic function and in allelic specificity, respectively. When the *Solanum chacoense* $S_{12}S_{14}$ genotype is transformed with an S_{11} RNase, the styles of plants expressing significant levels of the transgene reject S_{11} pollen. A previously characterized S RNase, S_{13}, differs from the S_{11} RNase by only 10 amino acids, four of which are located in the hypervariable regions. When $S_{12}S_{14}$ plants were transformed with a chimeric S_{11} gene in which these four residues were substituted with those present in the S_{13} RNase, the transgenic plants acquired the S_{13} phenotype. This result demonstrates that the S RNase hypervariable regions control allelic specificity.

INTRODUCTION

Self-incompatibility (SI) is the inherited ability of a fertile hermaphrodite flowering plant to prevent self-fertilization by discriminating between self- and non-self-pollen (de Nettancourt, 1977). SI thus constitutes a prefertilization barrier inhibiting pollen tube growth on or inside the pistil and preventing the formation of zygotes. This phenomenon is widespread in angiosperms, for which two major systems are known. In the sporophytic system, mainly studied in the Brassicaceae, the diploid SI genotype of the pollen-producing plant is responsible for the pollen phenotype (Nasrallah et al., 1994). In the gametophytic system (GSI), widely studied in the Solanaceae, the phenotype of the pollen is determined by its own haploid genotype (Clarke and Newbigin, 1993; Matton et al., 1994; Dodds et al., 1996).

Genetic control of GSI in the Solanaceae lies at a single multiallelic *S* locus, whose only known gene encodes a ribonuclease, termed the S RNase. This enzyme, which is expressed mainly in the transmitting tissue of the style (Anderson et al., 1986; Cornish et al., 1987; McClure et al., 1989, 1990), causes the arrest of pollen tube growth within the style. Gain-of-function experiments have shown that the introduction of a given S RNase confers that specific SI phenotype to the styles of the recipient transgenic plants (Lee et al., 1994; Murfett et al., 1994) and that the RNase activity is essential for the rejection of self-pollen (Huang et al., 1994). In these experiments, the pollen phenotype of the plants expressing the S RNase transgene remained unaltered (Lee et al., 1994). The nature of the pollen component, however, although presumably closely linked to the S RNase at the *S* locus, still remains one of the major enigmas in GSI.

A second puzzling feature of SI is associated with the large number of alleles in natural populations. Indeed, the genes encoding S RNases are among the most polymorphic plant genes known. Their products have amino acid sequence identity ranging from 35 to 96%. The mechanism(s) whereby new *S* alleles are generated, however, is not understood. On the one hand, new SI phenotypes have never been detected after classical mutagenesis (de Nettancourt, 1977). This has been interpreted as an inability of single amino acid replacements to generate new *S* alleles and has led to the proposal that new *S* alleles are generated by extensive rearrangements in gene structure (Pandey, 1972) or by gene conversion (Ebert et al., 1989). On the other hand, S RNases are encoded by single-copy genes, and the fact that they share a characteristic structure of five conserved and two hypervariable (HVa and HVb) regions (Ioerger et al., 1991) suggests that an accumulation of point mutations may be involved in generating new alleles. If true, then it should be possible to generate an S RNase with an altered specificity by site-directed mutagenesis, although it is likely that several amino acids must be simultaneously altered to account for the results of classical mutagenesis. The most likely targets for mutagenesis are the HV regions, which are believed to form a domain involved in allele-specific pollen recognition (Kao and McCubbin, 1996).

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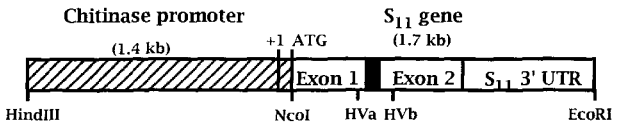
In the framework of studies on GSI, we have undertaken experiments to assess the role of the HV regions in allelic specificity in the wild tuber-bearing *Solanum chacoense*. This species is a true diploid that flowers abundantly and has a gametophytic system of incompatibility (Pal and Pushkarnath, 1944). Among the *S* alleles characterized thus far, the S_{11} and S_{13} alleles are the most similar *S* alleles known to be phenotypically distinct (Despres et al., 1994; Saba-El-Leil et al., 1994). They encode mature proteins that differ by only 10 amino acids, four of which are located in the HV regions. In addition, tester stocks are available for genetic analyses, and extensive breeding leading to the selection of plants with exceptionally high regeneration capacity from leaf discs has been conducted (Birhman et al., 1994; Van Sint Jan et al., 1996).

In this report, we show that transgenic plants harboring a chimeric S_{11} RNase gene modified only in its HV regions to mimic the S_{13} RNase gene have indeed acquired a full S_{13} phenotype. This demonstrates that one allelic form of the S RNase molecule can be converted into another by modification of the HV regions alone and that allelic specificity can be determined by the HV regions alone.



	HVa region	HVb region
S_{11} wt	KPKL**TY**N**Y**FSDKML	IDQASA**R**KDQP
S_{13} wt	....N.KF.....	.....L....

Figure 1. Schematic Diagram of the S_{11} and S_{11}–HVab Constructs.

The entire coding sequence of the S_{11} RNase gene or the S_{11}–HVab mutant was placed downstream of the style-specific tomato chitinase promoter. The HindIII-NcoI promoter fragment (hatched box) also contained the 5′ untranslated region of the chitinase gene. The genomic S_{11} RNase gene has an NcoI site engineered at the ATG translation initiation codon, a small (87-bp) intron (black box) between the HV regions HVa and HVb, and a 1-kb 3′ untranslated region (UTR). The amino acid sequences of the HV regions for the S_{11} and S_{13} wild-type (wt) alleles and the substitutions introduced to generate the S_{11}–HVab mutant are also shown. The dots indicate identical amino acids. Boldface residues indicate the four amino acids that differ in the HV regions of the S_{11} and S_{13} wild-type alleles.

RESULTS

Design of HV Domain Swap Mutants

We have recently isolated from two diploid self-incompatible but fully intercompatible lines of *S. chacoense* (PI458314 containing S_{11} and S_{12} alleles and PI230582 carrying S_{13} and S_{14} alleles) two phenotypically distinct *S* alleles (S_{11} and S_{13}). The sequences of their products differ by only 10 amino acids (Saba-El-Leil et al., 1994). Because four of these amino acid changes lie in the HV domains, these two alleles are ideally suited to evaluate the role of the HV domains with regard to *S* allele specificity in the context of a relatively constant protein backbone.

The gene constructs used to assess the role of the HV domain are shown in Figure 1. The constructs are based on a wild-type genomic S_{11} gene that has been placed under control of a strong pistil-specific chitinase promoter (Harikrishna et al., 1996). This promoter was chosen because (1) it had been successfully used to express an S RNase in an interspecific tobacco hybrid (Murfett et al., 1994), (2) it drives high levels of chitinase gene expression in tomato with a temporal and spatial pattern similar to that of the S RNases (Harikrishna et al., 1996), and (3) a chitinase gene whose product shares 92% amino acid sequence identity with the tomato chitinase gene is strongly expressed in styles of *S. tuberosum* (Wemmer et al., 1994). In addition, the close relationship between tomato and potato suggested that the promoter would work well in *S. chacoense*. The S_{11}–HVab mutant differs from the S_{11} wild-type product by the replacement of four amino acids in the HV domains with the corresponding amino acids from the product of the S_{13} wild-type allele. The constructs were introduced into a self-incompatible $S_{12}S_{14}$ *S. chacoense* genotype previously selected for high regenerability (Van Sint Jan et al., 1996). Transgenic plants were raised to maturity and brought to flowering to analyze the pollen rejection phenotype.

Pollen Rejection Phenotype Depends on the HV Domains

The breeding behavior of the transgenic plants was assessed by genetic crosses using pollen from $S_{11}S_{12}$ and $S_{13}S_{14}$ *S. chacoense* tester plants. In these crosses, compatible pollen tubes reach the ovary and fertilization occurs, which results in fruit formation. Incompatible pollen tubes, on the other hand, are arrested in the style, and no fruits are formed. The tester pollen stocks, which also contain either S_{12} or S_{14} alleles, are fully rejected by the endogenous $S_{12}S_{14}$ alleles of plants carrying the transgenes and do not contribute in any way to fruit formation.

As the results presented in Table 1 show, the presence of the S_{11} transgene conferred the ability to fully reject S_{11} pollen in the styles of 27% (18 of 66) of the transgenic plants. The remaining plants either partially rejected (11 of 66 plants) or completely accepted (37 of 66 plants) S_{11} pollen. The mature styles of all regenerated plants were also assayed by tissue printing to rapidly screen for transgene expression (Cappadocia et al., 1993), and only those displaying a positive phenotype had levels of transgene ex-

pression high enough to be measurable by this technique (Figure 2A). None of the plants randomly selected from those expressing the transgene rejected S_{13} pollen, as expected (Table 1), and none was self-compatible (data not shown).

Of the S_{11}–HVab plants, 36% (14 of 39) completely rejected and 20% (eight of 39) partially rejected S_{13} pollen. Because these plants could no longer completely reject S_{11} pollen, we conclude that the substitution of four amino acids in the HV domains is sufficient to confer the S_{13} phenotype to an otherwise S_{11} RNase. Again, only plants with the newly acquired S_{13} phenotype had levels of transgene expression high enough to be detected by tissue printing (Figure 2B). In addition, all of these transgenic plants were self-incompatible and fully rejected the pollen from the untransformed host genotype as well as from an $S_{12}S_{12}$ tester stock (data not shown).

Based on the genetic crosses (Table 1) and on levels of RNA expression, as determined by tissue printing (Figures 2A to 2C), we conducted further analyses for a selected group of plants, including individuals either expressing or not expressing the new phenotype. As shown in Figure 3, DNA gel blot analysis confirms that all plants studied contained from one to four copies of the transgene.

S_{11} Transgene Expression Results in a Gain-of-Function Phenotype

Slot blot analysis, performed with RNA extracted from mature styles of individual plants, shows a wide range of transgene expression (Figure 4). High levels of RNA expression were always observed in plants showing complete rejection of S_{11} pollen, whereas plants with low (T-12) or undetectable (T-57) levels of expression were either partially or fully compatible with S_{11} pollen, respectively. RNA gel blot analyses (data not shown) confirmed the results of the slot blots with respect to the amounts of the transgene transcript and also confirmed that only one transcript of the expected 1 kb was present. We conclude from these results that the newly acquired incompatible phenotype is only observed when steady state levels of transcript exceed a threshold amount, as was shown previously for petunia (Lee et al., 1994).

Complete Conversion of the S_{11}–HVab Mutant to an S_{13} Phenotype

As shown for the S_{11} gain-of-function phenotype, plants expressing variable levels of the S_{11}–HVab mutant transgene showed variable degrees of S_{13} pollen rejection (Figure 5). Again, plants with high levels of expression were able to fully reject S_{13} pollen, confirming that threshold levels of transcript appear to be required for the acquisition of the new phenotype. Plants expressing the mutant *S* allele were now unable to recognize and reject S_{11} pollen, showing that a complete conversion from the S_{11} to the S_{13} phenotype had occurred. Once again, individuals with low (T-4) or undetectable (T-7) levels of expression were unable to fully reject either type of pollen.

Transgene Expression Results in Functional RNase Activity

To verify that high levels of transgene expression resulted in production of the S RNase, we used two-dimensional electrophoresis to analyze the basic proteins present in the style (Sassa et al., 1993). All transgenic plants contained proteins corresponding to the RNases produced by the endogenous S_{12} and S_{14} alleles (Figure 6). In addition, the plants that acquired a gain-of-function phenotype resulting from transgene expression contained an additional protein whose electrophoretic mobility was identical to that of the S RNase produced by the authentic S_{11} allele. This protein is absent (T-57) or present in low amounts (T-7) in transgenic plants that do not express the transgene and do not show an altered phenotype. As predicted from their sequences, the

Table 1. Phenotypes of Transgenic Plants Assessed by Fruit Set after Pollination

	Transgenic Plant Phenotype					
	× $S_{11}S_{12}$ Pollen Donor[a]			× $S_{13}S_{14}$ Pollen Donor[a]		
Pistil Genotype $S_{12}S_{14}$	Comp	Partial Comp	Incomp	Comp	Partial Comp	Incomp
With S_{11} transgene	37[b]	11	18	16[c]	0	0
(66 plants)	(374/380)[d]	(47/216)	(0/418)	(117/124)		
With S_{11}–HVab transgene	36	3	0	17	8	14
(39 plants)	(336/341)	(23/32)	0	(142/153)	(37/78)	(0/135)

[a] Comp, compatible; Incomp, incompatible.
[b] Number of plants.
[c] These plants were selected randomly from the 29 plants showing a fully incompatible or partially compatible S_{11} phenotype.
[d] Number of fruits per number of pollinated flowers.

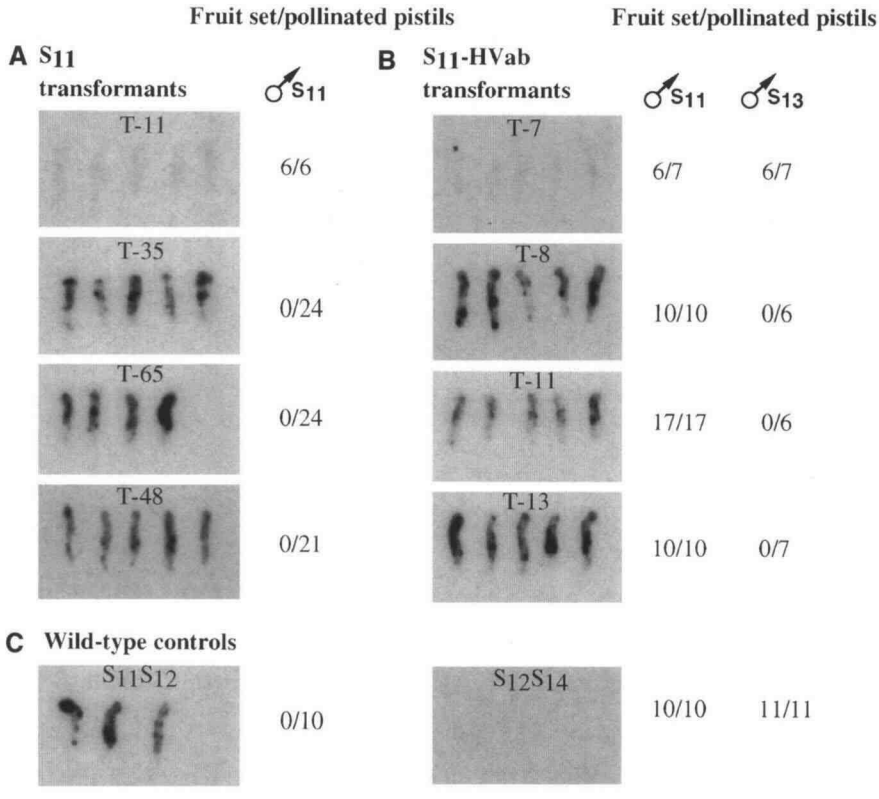


Figure 2. Selection of Gain-of-Function Phenotypes in Transgenic Plants.

(A) Tissue print analysis of S_{11} transgenic plants. For each transgenic plant tested, four or five styles were squashed on a charged nylon membrane, hybridized with an S_{13} cDNA clone, and exposed to film for 7 days. T-11 shows very low signal and no gain-of-function phenotype (six fruits produced from six pollinated flowers). Plants T-35, T-65, and T-48 show high levels of RNA expression and had no fruit set after pollination (i.e., a full rejection of S_{11} pollen).

(B) Tissue print analysis of the S_{11}–HVab mutant transgenic plants. Conditions were as described for **(A)**. Plant T-7 has very low signal after autoradiography and no gain-of-function phenotype. Plants T-8, T-11, and T-13 show strong RNA expression and acquired an ability to fully reject S_{13} pollen (no fruit set after pollination). These plants completely accepted S_{11} pollen.

(C) Tissue print analysis of wild-type $S_{11}S_{12}$ styles (positive control, three styles) and untransformed $S_{12}S_{14}$ styles (negative control, three styles).

proteins produced from the S_{11} and S_{11}–HVab mutant alleles have identical sizes and isoelectric points.

To verify that the transgene products had ribonuclease activity, in-gel RNase activity assays were performed (Yen and Green, 1991) (Figure 7). Plants expressing the new phenotype showed the presence of an active RNase corresponding in size to that found in wild-type S_{11} plants, whereas plants with neither RNA expression nor the appropriate SI phenotype lacked this RNase activity (T-57 and T-7).

S RNase Expression Results in Pollen Tube Growth Arrest

Microscopic examination of the stylar transmitting tissue isolated from transgenic plants 2 days after pollination clearly showed the arrest of pollen tube growth typical of an incompatible reaction. In plants with high levels of S_{11} expression, no S_{11} pollen tubes reached the ovary (Figure 8A, plant T-65), although these same plants let S_{13} pollen pass as expected (Figure 8B). In plants with intermediate levels of S_{11} transgene expression, only a few S_{11} pollen tubes could be observed to enter the ovary (Figure 8C, plant T-12).

In the case of plants expressing high levels of the S_{11}–HVab mutant transgene, S_{11} pollen was no longer recognized; thus, this type of pollen tube was allowed to reach the ovary and produce fruit (Figure 8E, plant T-13). These same plants, however, now completely rejected S_{13} pollen (Figure 8F). In plants expressing only intermediate levels of the S_{11}–HVab transgene, a few S_{13} pollen tubes reached the ovary (Figure 8D, plant T-4).

The appearence of pollen tubes in partially incompatible crosses (Figures 8C and 8D) differs from those in compatible crosses in that they appear brighter, because of a

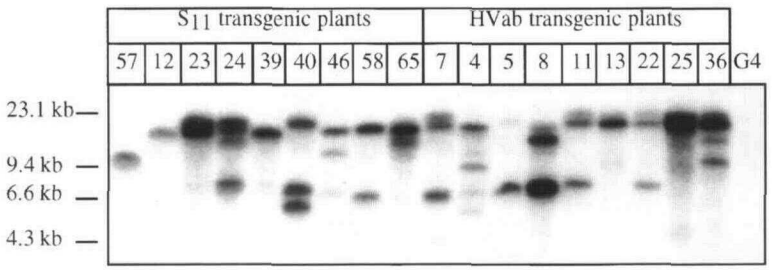


Figure 3. Transgene Copy Number as Determined by DNA Gel Blot Analyses.

DNA samples (10 μg) were digested with EcoRI, electrophoresed on agarose gels, and transferred to charged nylon membranes. After hybridization with the S_{13} cDNA probe, films were exposed for 3 days. Because there is only one EcoRI site in the construct, the number of bands seen on the gel corresponds roughly to the number of transgenes present. The untransformed $S_{12}S_{14}$ host plant (G4) does not show any hybridization signal, whereas all transformed plants (numbered above gel) show hybridizing bands. Molecular weight markers are shown at left.

heavier deposition of callose along their length, and lack the regularly spaced callose septa. It is possible that these features result from sublethal levels of the transgenic S RNase in the style.

DISCUSSION

Comparisons of all known solanaceous S RNase sequences have revealed a characteristic pattern of five highly conserved (designated C1 through C5) and two HV (termed HVa and HVb) regions (Ioerger et al., 1991). Based on this structure of the gene, it has been hypothesized that the HV regions are involved in the allelic specificity of the pollen rejection phenotype. Recent domain swap experiments in petunia and tobacco, designed to test this hypothesis, were unsuccessful (Kao and McCubbin, 1996; Zurek et al., 1997). In both cases, the chimeric S RNases retained their RNase activity but lost their ability to recognize their previously incompatible pollen without acquiring a new S phenotype. This led to the conclusion that the HV domains are necessary but not sufficient for conferring S specificity. However, the majority of the amino acid differences between the S RNases used in these domain swap experiments lie outside of the HV regions (36 of 52 for petunia, and 92 of 115 for tobacco). It is possible that in these chimerical gene products, interactions between the HV and constant regions have made the HV domains unable to display their normal allele-specific information. The failure to observe a new S phenotype could thus be due to the use of too highly divergent sequences, because compensatory mutations outside of the HV domains might be required to stabilize the interactions between the HV regions and the rest of the protein.

The characterization of the *S. chacoense* SI system has shown that the sequences of two of the alleles at the *S* locus (S_{11} and S_{13}) are quite similar (95% amino acid sequence identity for their products). Nonetheless, S_{11} and S_{13} RNases are phenotypically distinct with regard to their ability to recognize and reject their respective pollen. The similarity of the two alleles and the ability to introduce these alleles into a highly regenerable self-incompatible genotype whose endogenous *S* alleles ($S_{12}S_{14}$) do not interfere with the genetic and molecular interpretation of the transgene phenotype

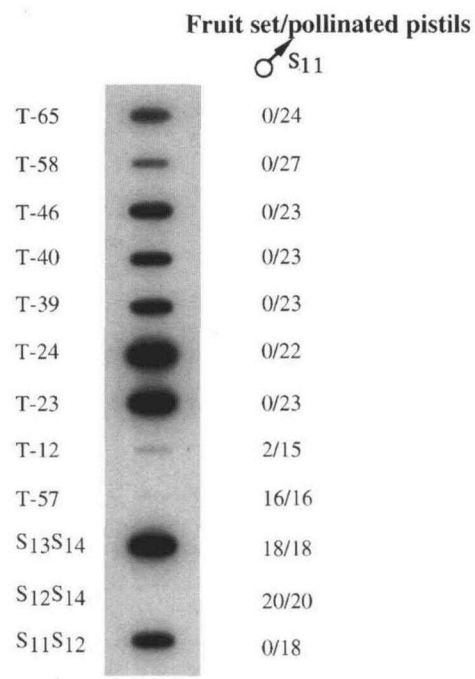
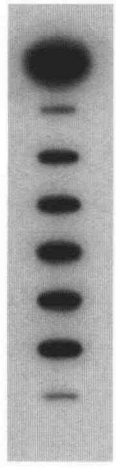


Figure 4. S_{11} Transgene Expression and Breeding Behavior for Selected Plants.

The amount of transgene expression was measured in 5 μg of total stylar RNA by probing slot blots with an S_{13} cDNA clone. As seen for the $S_{11}S_{12}$ genotype, the probe cross-hybridized with the S_{11} allele but not with the S_{12} or S_{14} alleles. Plants T-65, T-58, T-46, T-40, T-39, T-24, and T-23 show moderate to strong levels of transgene expression and completely rejected S_{11} pollen. Plant T-12 has weak levels of transgene expression and partially accepted S_{11} pollen. Plant T-57 has no transgene expression and completely accepted S_{11} pollen.



	Fruit set/pollinated pistils	
	♂S_{11}	♂S_{13}
T-36	9/10	0/20
T-25	9/9	0/6
T-22	10/10	0/10
T-13	10/10	0/7
T-11	17/17	0/6
T-8	10/10	0/6
T-5	7/7	0/6
T-4	6/7	3/8
T-7	6/7	6/7

Figure 5. S_{11}–HVab Transgene Expression and Breeding Behavior for Selected Plants.

RNA levels were monitored as described in the legend to Figure 4. RNA from plants T-36, T-25, T-22, T-13, T-11, T-8, and T-5 hybridized strongly with the S_{13} probe. These plants completely rejected S_{13} pollen while accepting S_{11} pollen. Plant T-4 has weak levels of transgene expression and partially accepted S_{13} pollen, whereas plant T-7 has very low levels of transgene expression and completely accepted both S_{11} and S_{13} pollen.

thus constitute a powerful system in which the determinants of allelic specificity can be dissected.

The initial step in our experiments was to develop a system in which gain of function could be demonstrated in plants harboring an S_{11} transgene. In a first series of experiments, plants were transformed with genomic fragments of the S_{11} allele containing either 1.4 or 0.8 kb of the putative promoter region. However, these constructs failed to express the S_{11} RNase gene (D.P. Matton, D. Morse, and M. Cappadocia, unpublished data), suggesting that additional positive *cis*-regulatory elements were lacking. Similar results were obtained previously with *Nicotiana* spp, in which neither 1.6 kb of the upstream promoter region from the S_2 nor 2.8 kb of the upstream promoter region from the S_6 *N. alata* alleles was sufficient to express the *S* transgene to wild-type levels (Murfett et al., 1995).

In the next series of experiments, the authentic *S* gene promoter was replaced with the promoter from the tomato chitinase gene (Harikrishna et al., 1996) that efficiently expressed the *S* RNase transgene, as shown by the large proportion of our transgenic plants that acquired the proper phenotype (Table 1). To fully document the gain-of-function phenotype, we analyzed the DNA, RNA, and proteins of transgenic plants as well as RNase activity and the ability to reject pollen. Gain of function was found to depend on a threshold level of transgene expression, as has also been shown for gain-of-function S phenotypes in petunia (Lee et al., 1994).

The molecular mechanism of pollen recognition by the stylar S RNases has been proposed to depend on the identity of the amino acids in the HV regions, because these are the regions in which most of the variation associated with allelic differences is found (Ioerger et al., 1991). These regions are hydrophilic and are thus likely to be found on the surface of the protein. Indeed, given the sequence similarity between the S RNases and RNase RH, for which the crystal structure is known (Kurihara et al., 1992), it is likely that the HV regions form a domain distinct from that containing the active site of the RNase. In this model, the amino acids that differ between the HV regions of the S_{11} and S_{13} alleles are all potentially exposed on the surface of a small part of the S RNase surface. It is thus reasonable to assume that these amino acids represent potential allele-specific recognition sites.

The S_{11}–HVab mutants generated and analyzed in this study showed a gain-of-function phenotype. As found for the S_{11} transgenic plants, individuals displaying the S_{13} phenotype all contained and expressed the S_{11}–HVab transgene. In addition, all produced a protein product that has

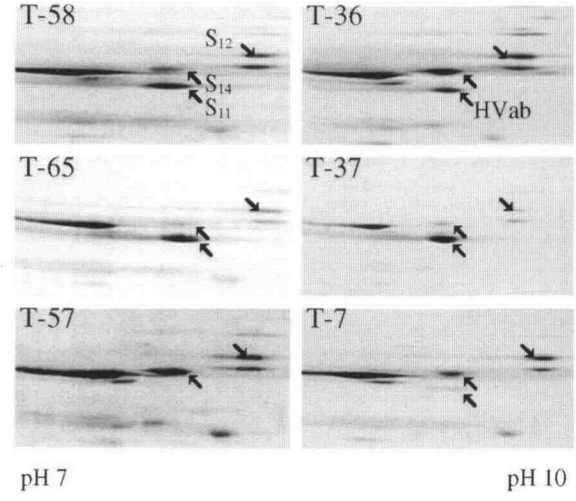


Figure 6. S RNase Protein Levels in Selected Plants.

Stylar S RNase levels were monitored by two-dimensional gel analysis of 50 μg of stylar protein. The identity of the S RNases shown was determined by their correlation with the alleles carried at the *S* locus in untransformed plants. Plants expressing the S_{11} transgene (T-58 and T-65) accumulated a protein corresponding in electrophoretic mobility to the authentic S_{11} protein in addition to the endogenous S_{12} and S_{14} proteins. Plants expressing the S_{11}–HVab transgene (HVab; T-36 and T-37) show the accumulation of a band corresponding in electrophoretic mobility to that expected for the S_{11}–HVab gene product together with the endogenous S_{12} and S_{14} proteins. Plants T-7 and T-57 expressed the transgene at low or undetectable levels, respectively.

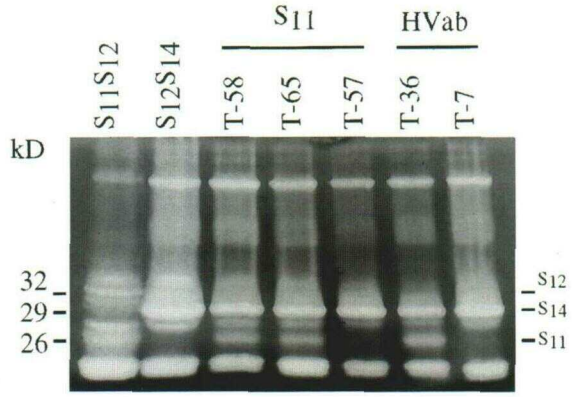


Figure 7. Transgene Products Have RNase Activity.

In-gel RNase activity staining of proteins extracted from the styles of control and transgenic plants shows that many bands of RNase activity are found in all of the plants. Only one of these bands, at a molecular mass of 26 kD, correlates with the presence of S_{11} at the *S* locus. Transgenic plants fully rejecting S_{11} pollen (T-58 and T-65) show this 26-kD RNase activity, as does the wild-type $S_{11}S_{12}$ genotype. A plant completely accepting S_{11} pollen (T-57) lacked this activity. A plant fully rejecting S_{13} pollen and expressing the S_{11}–HVab mutant transgene (T-36) shows an RNase activity at 26 kD, whereas plant T-7 fully accepting both S_{11} and S_{13} pollen lacks this activity.

the predicted electrophoretic mobility and RNase activity. However, plants expressing the S_{11} and the S_{11}–HVab transgene are different in their pollen rejection phenotype, with the S_{11}–HVab mutant blocking S_{13} pollen and no longer rejecting S_{11} pollen. Because the only amino acid replacements are found within the HV regions, whereas the rest of the protein retains its S_{11} character, we conclude that the HV regions are sufficient to confer allelic specificity on the S RNase. As proposed previously, the carbohydrate moiety of the S RNase glycoproteins does not seem to be involved in pollen-specific recognition (Karunanandaa et al., 1994).

The results shown here also provide a partial explanation regarding the number of amino acid differences that can be tolerated by an S RNase without a change in phenotype (Singh and Kao, 1992). The S_{11}–HVab mutant, which has acquired an S_{13} phenotype, encodes a protein that differs from the naturally occurring S_{13} RNase by only six amino acids distributed outside the HV regions. To date, this is the greatest number of amino acid differences found between two S RNases with an identical phenotype. In *Lycopersicon peruvianum*, two *S* alleles whose products share 98% amino acid sequence identity have been described, although it is still not known whether their phenotypes are distinct (Rivers et al., 1993; Royo et al., 1994).

Generation of new *S* alleles has been observed after forced inbreeding (Pandey, 1970) but never after classical mutagenesis (de Nettancourt, 1977). This has led to the suggestion that new *S* alleles are generated by extensive rearrangements in gene structure rather than an accumulation of

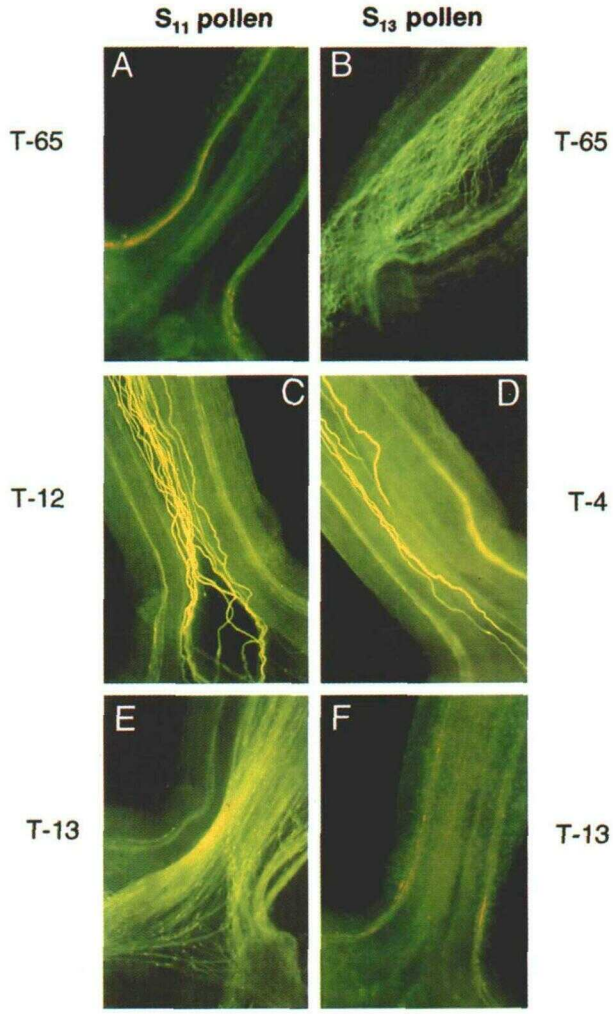


Figure 8. Microscopic Examination of Transgenic Plant Styles.

Shown are squashes of stylar bases stained with aniline blue and observed by epifluorescence 48 hr after pollination. The base of a squashed style is ~1 mm across, and all photographs are shown at the same scale. Pollen used in **(A)**, **(C)**, and **(E)** was derived from an $S_{11}S_{12}$ tester stock; pollen used in **(B)**, **(D)**, and **(F)** was derived from an $S_{13}S_{14}$ tester.

(A) and **(B)** Styles from plant T-65, which expressed the S_{11} transgene at high levels. These styles fully rejected S_{11} pollen and were perfectly compatible with S_{13} pollen, as can be seen by the numerous green fluorescent pollen tubes that enter the ovary.

(C) and **(D)** Plants T-12 and T-4 expressing intermediate levels of the S_{11} and S_{11}–HVab transgenes, respectively, with only a few pollen tubes being seen to reach the ovary. The pollen tubes appear more heavily stained because of increased callose deposition.

(E) and **(F)** Styles from plant T-13, which expressed the S_{11}–HVab construct at high levels. In these styles, S_{11} pollen was fully accepted, whereas S_{13} pollen was completely rejected.

point mutations (Ebert et al., 1989). Our results indicate that point mutations can lead to the formation of new *S* alleles, because a mutation of only 5 bp (four amino acids) results in a new phenotype. In this regard, it is interesting to note that chimeric alleles at the *b* incompatibility locus of *Ustilago maydis* that encode proteins differing by as few as two amino acids have been shown to display different specificities (Yee and Kronstad, 1993). In addition, a single amino acid difference can produce an altered incompatibility phenotype in *Podospora* spp (Deleu et al., 1993). Why then were new *S* alleles not detected after chemical mutagenesis? One possibility is that more than one mutational event in the allele may be required to generate a distinct phenotype, and chemical mutagenesis would be unlikely to produce such a mutant (Ramulu, 1980).

Although the results presented here shed some light on the molecular determinants of allelic recognition, several questions remain to be answered. For example, what is the minimum number of amino acid substitutions necessary and sufficient to confer an altered recognition phenotype to the *S* allele? Which amino acids are involved in determining allelic specificity and which are not? What are the recognition characteristics of S_{11} alleles in which only parts of the HV domains have been modified to resemble S_{13} alleles? The system described here seems well suited to provide unambiguous answers to these questions.

METHODS

Construction of a Chimeric S_{11} RNase Gene and Hypervariable Region Mutants

The entire coding sequence of the S_{11} RNase gene and its mutated form was placed downstream from the style-specific tomato chitinase (*Chi2;1*) promoter (Harikrishna et al., 1996). The HindIII-NcoI fragment from the chitinase promoter also contained the 5′ untranslated region of the chitinase gene. The S_{11} RNase gene was engineered to have an NcoI site containing the ATG translation initiation codon. All constructs also contained 1.0 kb of the S_{11} 3′ untranslated region.

The S_{11} gene was subcloned into the pALTER-1 mutagenesis vector from its original genomic 2.5-kb EcoRI fragment. Mutants were obtained through site-directed mutagenesis (altered sites II in vitro mutagenesis system; Promega) using the following mutagenic oligonucleotides (mutated nucleotides are underlined): HVa mutant primer, 5′-GCCAAAACTTAATTATAAATTTTTCAGTGT-3′; HVb mutant primer, 5′-GCTTCTGCTCTAAAGGACCAACC-3′. A mutagenic oligonucleotide containing the NcoI site (5′-TGCACCATGGTTAAATCACTGCTTAC-3′) was used in conjunction with an oligonucleotide complementary to the 3′ end of the gene (5′-GAATTCAAGGACATACATTTG-3′) to amplify the 1.7-kb S_{11} and the S_{11}–HVab mutant fragment by using polymerase chain reaction (Pwo DNA polymerase; Boehringer Mannheim). The 3.1-kb HindIII-EcoRI chimera was cloned in the transformation vector pBIN19 and introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. *Solanum chacoense* plants ($S_{12}S_{14}$ genotype) were transformed by the leaf disc method (Horsch et al., 1985). Each construction was sequenced before and after transformation in *Agrobacterium* to ensure the integrity of the NcoI junction and the mutated regions.

RNA and DNA Analyses

Total RNA was prepared as described previously (Jones et al., 1985). The RNA was quantitated spectrophotometrically and double-checked by ethidium bromide staining of agarose gels after electrophoresis of RNA samples denatured with methylmercury hydroxide (Sambrook et al., 1989). Tissue printing analysis was performed as described previously (Cappadocia et al., 1993). RNA slot blotting with 5 μg of total RNA and gel blotting with 10 μg of DNA were performed as described previously (Sambrook et al., 1989).

S RNase Electrophoresis and In-Gel Activity Assays

Proteins were extracted from the styles of flowers that had been open for 1 or 2 days (Lee et al., 1994). The styles were immediately frozen on dry ice and stored at −80°C until use. For extraction, 20 mg of material was ground to a fine powder in liquid nitrogen, and 0.4 mL of extraction buffer (50 mM Tris-HCl, pH 8.5, containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM $CaCl_2$, 1 mM DTT, and 1.25 mg/mL polyvinylpolypyrrolidone) was added. The extract was centrifuged for 15 min at 12,000*g*, and the supernatant was collected and stored at −80°C.

Protein concentrations were determined by Coomassie Brilliant Blue R 250 staining (Bradford, 1976). For SDS-PAGE of RNases, 15 μg of protein in standard sample buffer was electrophoresed on 12.5% polyacrylamide gels (Mini-Protean II; Bio-Rad), and the RNase activity was stained in the gel (Yen and Green, 1991). For two-dimensional gel electrophoresis, the supernatant was first precipitated in 80% (w/v) cold acetone and then dissolved in UKS buffer (Damerval et al., 1986) containing 1.6% (w/v) pH 3/10 and 3.2% (w/v) pH 8/10 ampholytes (Bio-Rad). Fifty micrograms of protein was subjected to nonequilibrium pH gradient electrophoresis, as described previously (Sassa et al., 1993), using a Mini 2-D cell (Bio-Rad). The second-dimension polyacrylamide gels were electrophoresed as described above and silver stained (Wray et al., 1981). The stylar proteins corresponding to the different *S* alleles were identified by correlation to plant genotypes, using the two parental lines, their F_1 progeny, and microspore-derived (homozygous) plants.

Genetic Crosses and Pollen Tube Staining

Genetic crosses were performed using pollen from tester stocks of known *S* allele constitution. Fully compatible crosses resulted in fruit formation after almost every pollination, whereas fully incompatible crosses never resulted in fruit set. Plants were scored as partially incompatible when fruit set lay between these two extremes.

Pollen tube growth within the style was observed using aniline blue fluorescence staining (Martin, 1959). Forty-eight hours after pollination, whole flowers were fixed in an ethanol–acetic acid solution (3:1) for 24 hr. The flowers were then immersed in 8 N NaOH for 1 hr at 60°C and rinsed thoroughly with tap water, and the pistils were isolated and squashed in a 0.1% (w/v) aniline blue solution in 0.15 M K_3PO_4. Pollen tubes were observed by epifluorescence microscopy using a Leitz Ortholux II microscope (Ernst Leitz GMBH, Wetzlar, Germany) equipped with a G filter (excitation: 350 to 460 nm).

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