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Production of an S RNase with Dual Specificity Suggests a Novel Hypothesis for the Generation of New *S* Alleles

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Gametophytic self-incompatibility in plants involves rejection of pollen when pistil and pollen share the same allele at the *S* locus. This locus is highly multiallelic, but the mechanism by which new functional *S* alleles are generated in nature has not been determined and remains one of the most intriguing conceptual barriers to a full understanding of self-incompatibility. The S_{11} and S_{13} RNases of *Solanum chacoense* differ by only 10 amino acids, but they are phenotypically distinct (i.e., they reject either S_{11} or S_{13} pollen, respectively). These RNases are thus ideally suited for a dissection of the elements involved in recognition specificity. We have previously found that the modification of four amino acid residues in the S_{11} RNase to match those in the S_{13} RNase was sufficient to completely replace the S_{11} phenotype with the S_{13} phenotype. We now show that an S_{11} RNase in which only three amino acid residues were modified to match those in the S_{13} RNase displays the unprecedented property of dual specificity (i.e., the simultaneous rejection of both S_{11} and S_{13} pollen). Thus, $S_{12}S_{14}$ plants expressing this hybrid S RNase rejected S_{11}, S_{12}, S_{13}, and S_{14} pollen yet allowed S_{15} pollen to pass freely. Surprisingly, only a single base pair differs between the dual-specific *S* allele and a monospecific S_{13} allele. Dual-specific S RNases represent a previously unsuspected category of *S* alleles. We propose that dual-specific alleles play a critical role in establishing novel *S* alleles, because the plants harboring them could maintain their old recognition phenotype while acquiring a new one.

INTRODUCTION

Among the cell–cell recognition phenomena present in living organisms, self-incompatibility (SI) plays a major evolutionary role because it constitutes an important mechanism for preventing inbreeding. SI is present in hermaphroditic animals such as tunicates (Grosberg, 1988), in fungi (Kronstad and Leong, 1990), and in many Angiosperm families (de Nettancourt, 1977). In the most widespread type of SI, gametophytic SI, the genotype of the haploid pollen determines its own incompatibility phenotype. For the Solanaceae, the gametophytic SI phenotype is specified by a highly multiallelic *S* locus (de Nettancourt, 1977, 1997) whose only known product is a ribonuclease (S RNase; McClure et al., 1989) expressed in the transmitting tissue of the style (Anderson et al., 1986). Gain-of-function experiments have shown that expression of an S RNase transgene is sufficient to alter the SI phenotype of the pistil but not that of the pollen (Lee et al., 1994; Murfett et al., 1994; Matton et al., 1997), and thus the identity of the pollen *S* gene (unknown to date) is likely to be different from that of the S RNase (Kao and McCubbin, 1997). RNase activity, although essential for expression of the SI phenotype (Huang et al., 1994), seems not to be involved in the specificity of the cell–cell recognition phenomenon. In closely related S RNases, such specificity has been shown to depend on the amino acid sequence at the two hypervariable regions (HVa and HVb) (Matton et al., 1997), whereas in distantly related S RNases, a role for amino acids located elsewhere in the molecule (in addition to the hypervariable regions) has been suggested (Kao and McCubbin, 1997; Zurek et al., 1997).

Multiallelism at the *S* locus is impressive in both sporophytic and gametophytic SI systems (de Nettancourt, 1997). In the sporophytic system, both point mutations and intragenic recombination appear to have contributed to *S* allele polymorphism (Kusaba et al., 1997; Nasrallah, 1997). In the gametophytic system, several studies (Clark and Kao, 1991; Coleman and Kao, 1992; Saba-El-Leil et al., 1994; Matton et al., 1995, 1997; Ishimizu et al., 1998) have suggested that point mutations rather than intragenic recombination (Fisher, 1961; Pandey, 1970; Ebert et al., 1989) are the primary source of *S* allele polymorphism. Phenotypically distinct yet highly similar S RNase sequences have been described in *Solanum chacoense* (Saba-El-Leil et al., 1994) and *Pyrus pyriflora* (Ishimizu et al., 1998).

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Studies of population dynamics predict that newly generated *S* alleles will spread in the population because of their reproductive advantage, until their frequency approaches that of other alleles. Several mechanisms to explain how new *S* alleles could be generated in nature have been proposed (de Nettancourt, 1977, 1997; Charlesworth, 1995). The major difficulty with these hypotheses is that the generation of new *S* allele specificities, either in the pollen or in the style alone, would inevitably result in the breakdown of the SI system. Only if appropriate changes in the pollen and stylar sequences occur concurrently can the SI phenotype be maintained.

By using site-directed mutagenesis to dissect the elements required for determining allelic specificity, we have produced a mutant that bears on the question of new *S* allele generation. Our experimental system, the tuber-bearing wild potato species *S. chacoense*, involves two phenotypically distinct *S* alleles (S_{11} and S_{13}) whose mature proteins differ by only 10 amino acids (Saba-El-Leil et al., 1994), four of which are located in the hypervariable regions (Figure 1B). We have shown that the substitution of all four of these amino acids in the hypervariable regions of an S_{11} RNase with those of an S_{13} RNase fully converts the S_{11} phenotype into an S_{13} phenotype (HVab S RNase; Matton et al., 1997). Here, we show that altering only three of these four amino acids (HVapb S RNase) can result in a new *S* allele with the unexpected property of dual-specificity incompatibility, or DSI (i.e., capable of rejecting simultaneously two phenotypically and genotypically distinct pollen types). *DSI* alleles, if produced in nature, could represent an essential step in the generation of new *S* alleles from preexisting ones in a mechanism that would prevent any breakdown of the SI system.

Although never before observed in the context of SI, dual specificity has been observed in other plant cell–cell recognition phenomena. For example, the disease resistance *RPM1* gene of Arabidopsis confers resistance to *Pseudomonas syringae*, expressing either the *avrRpm1* avirulence gene or the unrelated *avrB* pathogen signals (Grant et al., 1995). Furthermore, the polygalacturonase-inhibiting *pgip-2* gene of bean encodes a protein that interacts with and specifically inhibits the endopolygalacturonases from both *Aspergillus niger* and *Fusarium moniliforme* (Leckie et al., 1999). Dual specificity may be more common in cell–cell recognition systems than currently thought, and if so, it may play an important role in the evolution of new recognition specificities in general.

RESULTS

The *HVapb* Transgene Is Derived from a Partial Domain Swap between the S_{11} and S_{13} Alleles

We have previously shown that replacing the four amino acids that differ between the HVa and HVb regions of S_{11} and S_{13} RNases resulted in complete conversion of the S_{11} rejection phenotype into that of S_{13} (the *HVab* allele; Figure 1B) (Matton et al., 1997). Using a style-specific tomato chitinase promoter (Harikrishna et al., 1996) to drive expression of a hybrid S_{11} allele in vivo, we produced an S RNase (the HVapb RNase; Figure 1B) in which three of these four amino acids in the HVa and the HVb regions of the S_{11} RNase were replaced with those of the S_{13} RNase. Thus, the HVapb RNase differs in only one amino acid from the previously reported HVab RNase, which displays an exclusively S_{13} SI phenotype (Figure 1B).

Genetic Crosses Show That the *HVapb* Transgene Rejects Both S_{11} and S_{13} Pollen

The breeding behavior of G4 plants ($S_{12}S_{14}$ genotype) transformed with the *HVapb* transgene was fully assessed by genetic crosses repeated over two consecutive years using pollen from suitable tester stocks. In these crosses, an incompatible reaction is expected between the endogenous S_{12} and S_{14} RNases present in the styles of the transformed plants and S_{12} and S_{14} pollen when produced by G4 plants (whether transformed or not). Any other pollen types (S_{11} or S_{13}, for example) should experience no stylar barrier to fertilization and produce seeded fruits after pollination unless an additional incompatibility barrier is generated by expression of the transgene. The absence of fruits is a sensitive indicator of pollen rejection, because fruits can be set when even a few pollen tubes reach the ovary.

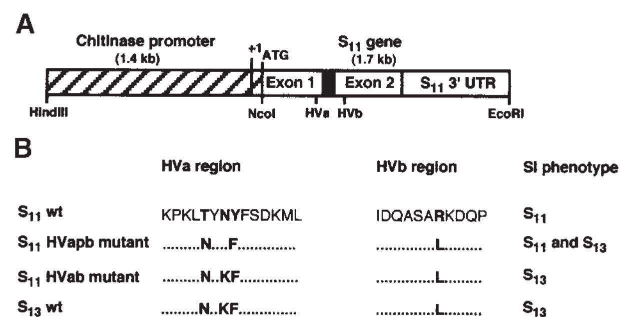

Figure 1. Structure of the *HVapb* Transgene Construct.

(A) The chimeric transgene contains the entire coding sequence of a mutated S_{11} gene downstream from a style-specific tomato chitinase promoter (Harikrishna et al., 1996). The hatched box corresponds to the chitinase promoter and includes the chitinase 5′ untranslated region (UTR). The black box corresponds to the S_{11} intron.
(B) The amino acid sequences in the hypervariable regions HVa and HVb of the S_{11} wild-type allele are shown with the residues targeted for site-directed mutagenesis in boldface. Dots represent unchanged amino acids. The residues differing in the hypervariable regions of the S_{13} wild type (wt) and the previously described *HVab* mutant (Matton et al., 1997) are shown for comparison along with their associated phenotypes.

We found that seven individuals from a total of 33 different plants transformed with the *HVapb* transgene (21% of the transgenic plants) fully rejected S_{11}, S_{12}, S_{13}, and S_{14} pollen. This high number of plants with a new and complete SI phenotype is similar to that observed with other *S* transgenes in this species (27 to 36%; Matton et al., 1997) and is believed to be due to the strong chitinase promoter used in the gene constructs. The genetic crosses (Table 1) showed that these seven plants had acquired the ability to reject two phenotypically distinct pollens (S_{11} and S_{13}), despite being transformed with only one type of *S* transgene. This was most clearly seen in the simultaneous rejection of pollen produced from an $S_{11}S_{13}$ genotype in addition to pollen from plants containing either S_{11} or S_{13} in different genetic backgrounds (Table 1). The transgenic plants behaved normally with respect to their endogenous SI phenotype ($S_{12}S_{14}$), as shown by complete lack of fruit set after self-pollinations or crosses with pollen from the untransformed $S_{12}S_{14}$ genotype.

The possibility that the transgenic plants were female sterile was excluded by the observations that fruits were set after pollinations with (1) compatible 2n pollen from G4 tetraploids (data not shown) and (2) haploid pollen from an $S_{14}S_{15}$ individual (Table 1). These later crosses also ruled out the possibility that the transgenic plants could reject pollen with any *S* allele constitution. We conclude from these genetic data that the HVapb RNase is dual specific because it recognizes and rejects the phenotypically distinct S_{11} and S_{13} pollen yet does not block unrelated pollen types. These seven plants thus acquired a full DSI phenotype. The remaining plants were scored as either partially or fully compatible because they let pass S_{11} and S_{13} pollen to varying degrees (Table 1). As occurs with fully DSI plants, all retained their endogenous $S_{12}S_{14}$ phenotype.

To determine how the DSI phenotype was related to expression of the transgene, we first ranked transformed plants on the basis of *HVapb* RNA levels by tissue printing (Cappadocia et al., 1993). A selection of these tissue prints is shown in Figure 2. Typically, five different styles from each plant were printed onto nylon membranes and hybridized first to the S_{13} cDNA probe (Figures 2A to 2C, left) and then to an 18S rRNA probe as a control for RNA levels (right). The tissue prints are arranged so that those with the highest and lowest levels of *HVapb* RNA are at the top of Figure 2A and at the bottom of Figure 2C, respectively, whereas controls for the specificity of the S_{13} cDNA probe are shown at left in Figure 2D. The pollen rejection phenotype (i.e., the number of fruits set per number of pollinations performed) is shown for both S_{11} and S_{13} pollen beneath the tissue prints for each transgenic plant. As shown, the full DSI phenotype (full rejection of both S_{11} and S_{13} pollen) was associated with those plants expressing the highest levels of transgene RNA, whereas low levels of transgene expression are associated with a partially or fully compatible pollen rejection phenotype. Interestingly, some individuals with intermediate levels of transgene expression fully rejected S_{13} pollen but only partially rejected S_{11} pollen (e.g., plants T-11, T-21, T-6, and T-1). This is not surprising, given that the HVapb S RNase sequence differs from the S_{11} sequence by three amino acids and from the *HVab* transgene (an S_{13} rejection phenotype) by only one amino acid (Figure 1B). The additional 12 plants lacking a detectable phenotype (Table 1) also had no detectable RNA expression in these tissue prints (data not shown).

Molecular Characterization of the DSI Plants

To confirm that the acquisition of DSI was due to a single S RNase whose characteristics were those expected based on the *HVapb* gene sequence, we subjected five of the plants with a complete DSI phenotype (T-16, T-18, T-20, T-30, and T-31) to a detailed molecular analysis. One transgenic plant that had not acquired a new rejection phenotype (T-19) was included as a negative control. DNA gel blots using an S_{13} cDNA probe confirmed that all transformants contained at least one copy of the transgene and that all

Table 1. A Dual-Specificity Pollen Rejection Phenotype Is Revealed by Genetic Crosses

Pistil Genotype ($S_{12}S_{14}$)	No. of Plants	Pollen Donor Genotype $S_{11}S_{12}$	$S_{13}S_{14}$	$S_{11}S_{13}$	$S_{12}S_{14}$	$S_{14}S_{15}$
Incompatible[a]	7	0/155[b]	0/138	0/112	0/78	31/32
Partially compatible[c]	13	141/213	47/228	ND[d]	0/54	ND
Compatible[e]	13	215/224	137/139	12/12[f]	0/5[f]	8/8[f]
Untransformed host (G4)		14/14	17/17	15/15	0/20	5/6

[a] Crosses were scored as fully incompatible when pollinations never resulted in fruit formation.
[b] Number of fruits set per number of pollinated flowers.
[c] Plants were scored as partially compatible when several fruits were set after pollination.
[d] ND, not determined.
[e] Crosses were scored as fully compatible when almost every pollination resulted in fruit set.
[f] Data from only one plant (T-19).

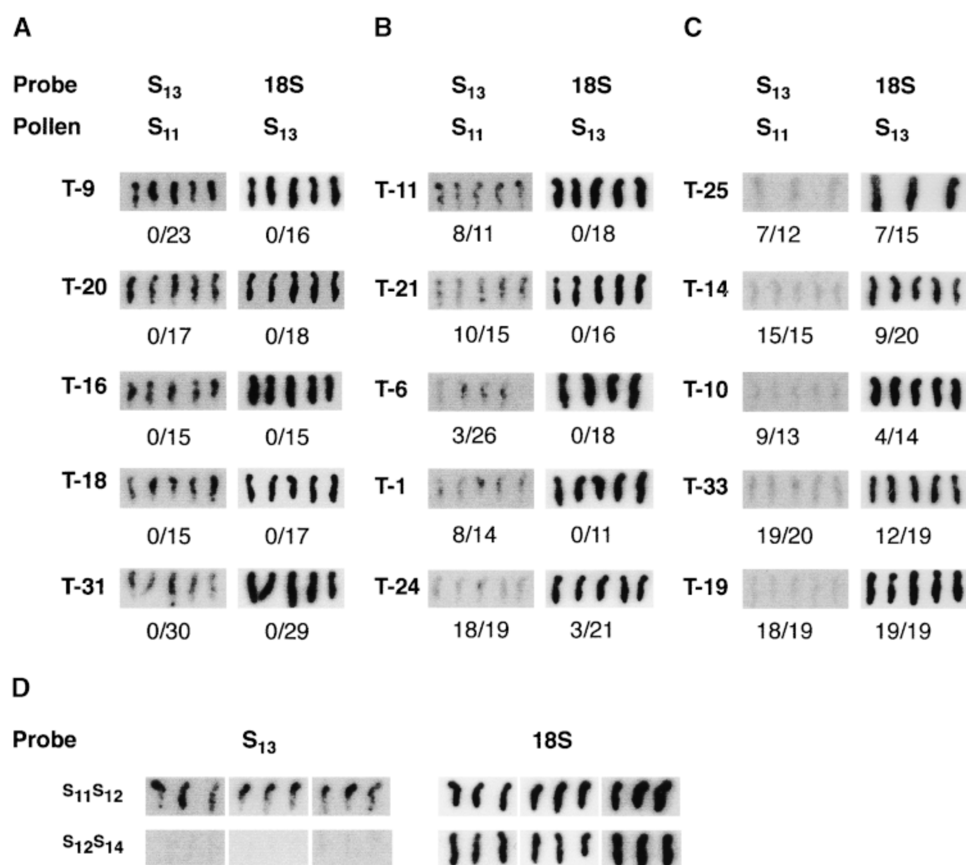

Figure 2. *HVapb* Transgene Expression and Breeding Behavior for Selected Plants.

(A) For each transgenic plant T-9, T-20, T-16, T-18, and T-31, four to five styles were squashed on a charged nylon membrane, hybridized with an S_{13} cDNA probe, and exposed to film for 7 days (blots at left for each plant). All hybridizations and exposures were performed at the same time for all plants. The membranes were then stripped, hybridized with an 18S rRNA probe, and exposed to film overnight (blots at right for each plant). The pollen rejection phenotypes (number of fruits set/number of pollinations) with either S_{11} (left) or S_{13} (right) pollen are shown immediately below the tissue prints for each plant to allow direct comparisons with the levels of transgene expression. No fruits set after pollination represents a full incompatible phenotype.

(B) Transgenic plants T-11, T-21, T-6, T-1, and T-24 were treated as described in **(A)**.

(C) Transgenic plants T-25, T-14, T-10, T-33, and T-19 were treated as described in **(A)**.

(D) Nine styles from either $S_{11}S_{12}$ (top) or $S_{12}S_{14}$ (bottom) genotypes were squashed on a charged nylon membrane, hybridized to an S_{13} cDNA probe (left), and exposed to film at the same time as for the transgenic plants. The membranes were then stripped and hybridized to an 18S rRNA probe (right).

transgenics were derived from independent transformation events (Figure 3). Control hybridizations with plants of known genotype confirmed that the probe hybridized readily to both S_{11} and S_{13} alleles. The transgene expression as assessed by RNA gel blot analyses (Figure 4A) confirmed that plants that had acquired the DSI phenotype expressed an mRNA whose length (1 kb) and amounts were similar to those observed in wild-type $S_{11}S_{12}$ or $S_{13}S_{14}$ plants. Hybridization with a constitutively expressed β-amylase probe as control (Figure 4B) showed that all samples contained similar amounts of RNA. Only a single size class of mRNA hybridized to the S_{13} cDNA probe in the transgenic plants.

Protein extracts from the styles of plants expressing the DSI phenotype were analyzed by gel electrophoresis. First, two-dimensional gel electrophoresis (Figure 5) showed that the transgenic plants T-16, T-20, T-30, and T-31 contained a protein found neither in the untransformed host plant ($S_{12}S_{14}$) nor in the control plant T-19. This new protein had an apparent molecular weight identical to that previously observed for transgenic plants expressing an authentic S_{11} transgene (Matton et al., 1997) but a slightly more acidic pI, consistent with the arginine-to-leucine substitution in the HVb region (Figure 1). To confirm that the new protein was similar in sequence to the S_{11} RNase, we probed protein gel

blots of stylar extracts with an antibody raised against a 15–amino acid peptide corresponding to the S_{11} HVa region. This antibody specifically recognizes the S_{11} RNase, because the S_{13} RNase did not cross-react (Figure 6A, left). The antibody did cross-react, however, with a protein in the stylar extracts of transformed plants that had acquired the DSI phenotype (Figure 6A). No reaction was observed with control plant T-19, despite a protein load similar to that in other lanes (Figure 6B). The amount of HVapb RNase is comparable in plants that acquired the DSI phenotype; plant T-30, which appeared to contain less immunoreactive protein, also contained less total protein, as shown by Ponceau red staining (Figure 6B). However, these protein blots cannot be used to compare levels of the HVapb RNase with the S_{11} RNase because these two proteins have a different sequence in the region of antibody binding (the HVa region; Figure 1B).

The immunoreactive protein generally began to accumulate in styles ~2 days before flower opening (Figure 6C), similar to the pattern of S_2 and S_3 RNase expression reported in this species (Xu et al., 1990). The accumulation of the S RNase can also be observed by general protein stains (Figure 6D). Because unopened flower buds can be self-pollinated and set fruit, the ability of the unopened buds of transgenic plants to reject S_{11} pollen was also tested at 3 and 2 days before bud opening. When styles of plant T-20 were observed by UV microscopy 30 hr after pollination, buds pollinated 3 days before opening showed numerous pollen tubes (>50) in the ovarian region, whereas buds pollinated 2 days before opening showed few (<5) pollen tubes (data not shown). The time at which these latter flower buds were examined thus correlated with the time detectable levels of the HVapb RNase were observed (1 day before bud opening). The presence of immunologically detectable levels of the HVapb RNase is thus a prerequisite for pollen rejection. By the time of flower opening (day 0), plant T-20 was fully incompatible with both S_{11} and S_{13} pollen (no pollen tubes reached the ovary), and the pollen rejection phenotype was microscopically identical to that of plant T-16 described below.

Lastly, when stylar protein extracts were tested for RNase activity by in-gel RNase assays, a new RNase activity whose electrophoretic mobility was identical to that of the authentic S_{11} RNase (expressed by transgenic plant T-64 S_{11}) (Matton et al., 1997) was observed (Figure 7, arrows). This activity was absent in the untransformed ($S_{12}S_{14}$) host plant or in plant T-19. This demonstrates that the transgene product has RNase activity. Taken together, we conclude from these data that the acquisition of the DSI pollen rejection phenotype is due to the expression of a single active S RNase whose molecular characteristics are those expected from the HVapb sequence.

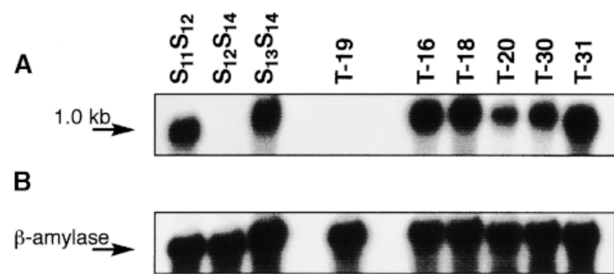

Figure 4. DSI Transgenic Plants Express Wild-Type Levels of mRNA.

(A) Ten micrograms of total stylar RNA was electrophoresed on agarose gels containing formaldehyde and transferred to nylon membranes. The membranes were hybridized with the S_{13} cDNA probe and exposed to film for 2 hr. The hybridization signal from five of the six transformed plants carrying the *HVapb* transgene was similar to S RNase transcript levels in the untransformed plants (Matton et al., 1997) (left lanes). The $S_{12}S_{14}$ genotype (G4) is the transformation host. The size of the hybridizing mRNA is 1 kb.

(B) The membrane used in **(A)** was rehybridized with a β-amylase gene probe and exposed to film for 3 hr to show that similar amounts of RNA were present in all samples.

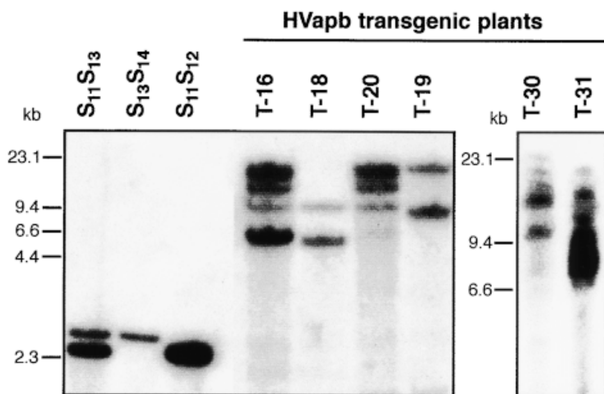

Figure 3. Transgenic Plants Contain from One to Four Copies of the Transgene.

Ten-microgram samples of genomic DNA were digested with EcoRI, electrophoresed on agarose gels, and transferred to nylon membranes. After hybridization with an S_{13} cDNA probe, films were exposed for 3 days. The S_{13} cDNA probe hybridizes with both S_{11} and S_{13} alleles in untransformed plants (leftmost three lanes) but does not recognize the S_{12} or S_{14} alleles in the host plant. Numbers at left show the position of molecular weight standards (in kilobases).

Simultaneous S_{11} and S_{13} Pollen Tube Growth Arrest in Styles of DSI Plants

As a final confirmation of the DSI phenotype, the styles of HVapb-expressing individuals were examined by UV microscopy 48 hr after pollination with pollen from an $S_{11}S_{13}$ individual. Both S_{11} and S_{13} pollen tubes, observed in the

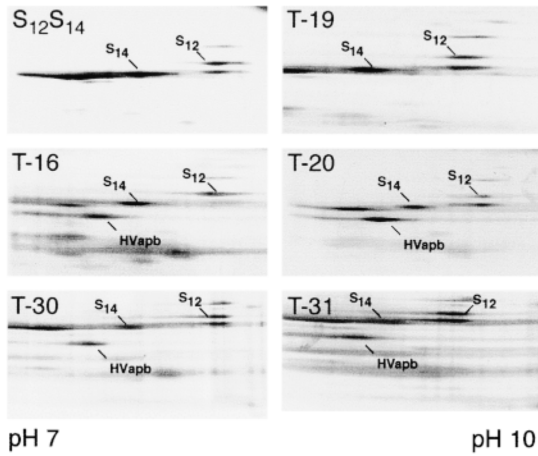

Figure 5. Two-Dimensional Gels Show That a New Protein Is Present in DSI Transgenic Plants.

Fifty micrograms of stylar protein was electrophoresed on two-dimensional gels and visualized by Coomassie Brilliant Blue R 250 staining. The spot labeled HVapb is not found in the untransformed host ($S_{12}S_{14}$) or in plants without a detectable phenotype (T-19). This new protein has the same molecular mass as the S_{11} RNase but a slightly more acidic pI, as expected based on the sequence modifications.

midstyle of plant T-16 (Figure 8C, upper right panel), had thickened callose walls, were few in number, and did not penetrate to the ovarian region (Figure 8C, bottom right). These characteristics are identical to those of a fully incompatible cross, such as when S_{12} and S_{14} pollen are observed in the styles of untransformed G4 plants (Figure 8A, right panels). This SI response to incompatible pollen was not observed when pollen from an $S_{11}S_{13}$ individual grew in the styles of untransformed (G4) plants (Figure 8A, left panels) or in the styles of plants not expressing the transgene (plant T-19; Figure 8C, left panels). In these fully compatible crosses, pollen tubes passed freely through the styles and entered the ovary.

DISCUSSION

Plants with the Full DSI Phenotype Express the *HVapb* Transgene

The DSI phenotype is the ability to completely reject two phenotypically distinct pollens (S_{11} and S_{13}) and was observed in seven of the 33 transgenic plants produced. This high number of plants acquiring a new SI phenotype is in good agreement with our previous results and is believed to result from the strong chitinase promoter used in the constructs (Matton et al., 1997). The conclusion that the new phenotype results from expression of the transgene is based on (1) expression of *HVapb* RNA in plants that have acquired the DSI phenotype (Figures 2 and 4), (2) expression of S RNase in plants that have acquired the DSI phenotype (Figures 5, 6A, and 7), (3) generally increased RNA levels in

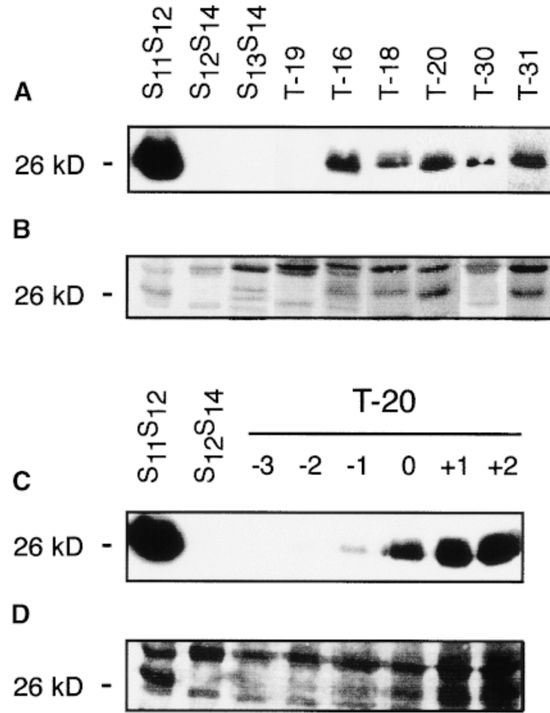

Figure 6. DSI Transgenic Plant Style Extracts Cross-React with an Anti-S_{11} RNase Antibody.

(A) Twenty micrograms of stylar extract prepared from buds at the time of flower opening (day 0) was electrophoresed on an SDS–polyacrylamide gel, transferred to nitrocellulose, and challenged with the anti-S_{11} antibody. Flowers were obtained from untransformed plants with known *S* allele constitutions, a transgenic plant lacking an observable phenotype (T-19), or DSI transgenic plants (T-16, T-18, T-20, T-30, and T-31). Each day 0 sample was taken from a time-series gel similar to that shown in **(C)**. To ensure equal development times, all gels contained samples from $S_{11}S_{12}$ and $S_{12}S_{14}$ plants as positive and negative controls, respectively. The DSI transgenic plants contain a protein similar in size to the authentic S_{11} RNase, which reacts with the antibody, although HVapb RNase levels cannot be directly compared with those of the S_{11} RNase due to the differences in their amino acid sequences in the region of antibody binding. The size of the HVapb RNase (26 kD) is shown at left.

(B) Ponceau-S staining of the nitrocellulose in **(A)** before immunoblotting.

(C) Twenty micrograms of stylar extract was prepared from buds harvested from plant T-20 at the indicated times (in days) of flower development (day 0 represents flower opening), electrophoresed, and subjected to immunoblotting as given in **(A)**. Films were exposed approximately twice as long as those shown in **(A)** to visualize the low levels of protein in unopened flowers.

(D) Ponceau-S staining of the nitrocellulose in **(C)** before immunoblotting.

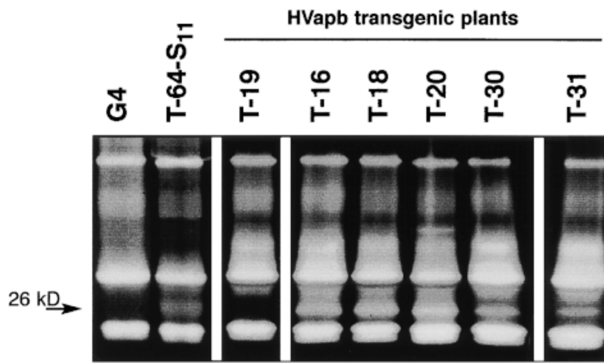

Figure 7. DSI Transgenic Plants Contain a New RNase Activity.

In-gel RNase activity assays using 50 μg of stylar protein (Yen and Green, 1991) show a band of 26 kD (arrow) present in extracts from transgenic plants expressing either an authentic S_{11} (T-64-S_{11}) or the HVapb transgene (T-16, T-18, T-20, T-30, and T-31). This band is absent in the untransformed G4 genotype and in plants without detectable transgene transcript (T-19).

plants with higher levels of pollen rejection (Figure 2), and (4) an increase in the pollen rejection phenotype with increasing HVapb RNase accumulation (Figure 6C).

The full DSI phenotype is typically associated with expression of the transgene at a level similar to endogenous S RNases for both RNA and protein. However, we find there are exceptions to this general rule. For example, plant T-20 has average levels of HVapb RNase yet lower than average levels of RNA (cf. Figures 4 and 6). Furthermore, plant T-18 has low levels of protein despite high levels of RNA (cf. Figures 4 and 6). These discrepancies may be related to differences in the stability of transgene products at both mRNA and protein levels in the independent transgenic lines, because our measurements reflect accumulated quantities (i.e., neither the synthesis nor the degradation rates of the S proteins or the *S* mRNAs are known). Furthermore, because transgene expression is developmentally regulated (Figure 6C), differences in sampling time or even in the timing of transgene expression could produce differences in the accumulated levels of mRNA or protein. Also, other factors, such as the effects of pollination (perhaps not negligible, given that the wound-inducible chitinase promoter drives our transgene) or temperature, may contribute to variations in the levels of molecular correlates to the phenotype.

Single Amino Acid Changes Can Alter the SI Phenotype

We have previously shown that full conversion of the S_{11} into the S_{13} pollen rejection phenotype was obtained by substituting the four residues in the hypervariable regions that differed between the S_{11} and S_{13} RNases (Matton et al., 1997). Here, we demonstrate that partial replacement of these four key amino acids can lead to the production of a functional S RNase with a DSI phenotype. A careful examination of the amino acids altered in the HVapb RNase (Figure 1), which rejects both S_{11} and S_{13} pollen, leads to two important conclusions concerning the relationship between S RNase sequence and the resulting incompatibility phenotype. First, a total of seven amino acid differences (six of which lie outside the hypervariable regions) separate the sequence of the HVapb RNase from the wild-type S_{13} RNase. Because both of these S RNases reject S_{13} pollen (Figure 1), this represents the largest number of amino acid changes described to date that can be accommodated by an S RNase without elimination of its pollen rejection capability. Second, when the sequence of the HVapb RNase is compared with that of the previously produced HVab RNase (Figure 1), only a single amino acid is found to differ between the two. This amino acid substitution (K→N), due to a single base pair transversion (A→C), is the only difference between the previously described S_{13}-specific *HVab* mutant (which does not reject S_{11} pollen; Matton et al., 1997) and the *HVapb* mutant (which does) (Figure 1). This thus represents the smallest difference between two S RNase sequences with a different pollen rejection phenotype and provides convincing experimental support for the argument that single point mutations

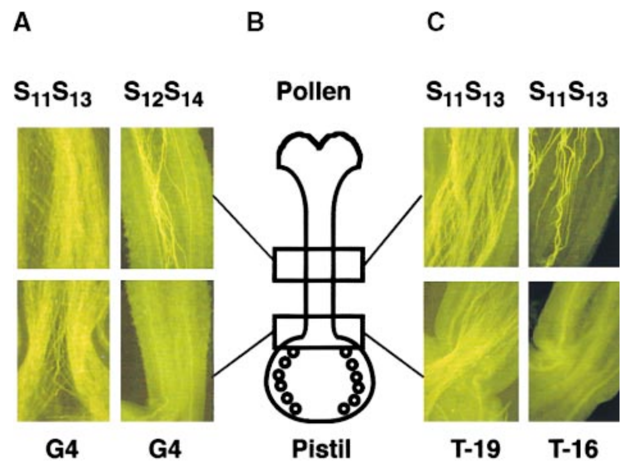

Figure 8. DSI Transgenic Plants Display a Typical Pollen Rejection Phenotype with Two Distinct Pollen Types.

(A) and **(C)** Squashes of midstyle (top) and stylar bases (bottom) of either untransformed G4 or transformed plants T-19 or T-16 were stained with aniline blue 48 hr after pollination (Martin, 1959) with pollen from either an $S_{11}S_{13}$ or an $S_{12}S_{14}$ tester stock. All pollen tube growth was arrested in plants expressing the *HVapb* transgene (T-16) but not in plants lacking detectable transgene expression (T-19). Controls show a fully compatible pollination of untransformed G4 plants with S_{11} and S_{13} pollen (left) and an incompatible pollination of G4 plants with self-S_{12} and self-S_{14} pollen.

(B) A schematic view of the pistil illustrates which regions of the styles were examined for the presence of pollen tubes.

can generate new *S* allele specificities (Clark and Kao, 1991; Saba-El-Leil et al., 1994).

The recognition characteristics of our antibody, directed against the HVa region of the S_{11} RNase (an anti-S_{11} antibody), also support the hypothesis that single amino acid changes can alter S RNase recognition. The anti-S_{11} antibody recognizes both S_{11} and HVapb RNases but does not recognize the S_{13} RNase. It is thus reasonable that the pollen component of the *S_{11}* allele, capable of binding an S_{11} RNase with an affinity similar to that of the anti-S_{11} antibody, should also be able to discriminate between the HVapb and the S_{13} RNases (which differ only in a single amino acid in the HVa region). We also note that the recognition of both S_{11} and HVapb RNases by our antibody is in agreement with the intermediate nature of the HVapb RNase. Because the anti-S_{11} antibody has a surface complementary to the S_{11} RNase, its behavior thus mimics in vitro the expected in vivo interaction between the pollen S component and its corresponding S RNase. Clearly, these specific reactions imply that the hypervariable regions of S RNases lie exposed at the surface of the protein, as proposed by recent three-dimensional models of RNase structure (Parry et al., 1998). As a caveat to any sweeping generalization that might be drawn from these observations, however, we note that the substitution of asparagine by lysine (which eliminates antibody binding as well as S_{11} pollen recognition) replaces an uncharged residue with a larger, positively charged residue. It remains to be determined whether less drastic single amino acid substitutions would also be sufficient to alter the SI phenotype and antibody binding.

A single amino acid change has recently been shown to alter the recognition specificity of the polygalacturonase inhibiting protein (PGIP) of bean. PGIP is encoded by a gene family, and the proteins produced by different family members recognize and inhibit polygalacturonases from different fungi with different specificities. For example, PGIP-1 inhibits polygalacturonases from *A. niger*, whereas PGIP-2 inhibits polygalacturonases from both *A. niger* and *F. moniliforme* (Leckie et al., 1999). The sequences of the monospecific PGIP-1 and the dual-specific PGIP-2, which differ by only eight amino acids, are analogous to our wild-type S_{13} and HVapb sequences. Interestingly, when the glutamine at position 253 of the PGIP-2 sequence is mutated to a lysine, the dual specificity of PGIP-2 is lost. Conversely, the monospecific PGIP-1 can be converted to dual specificity by replacement of the lysine at position 253 by glutamine. This behavior is clearly similar to our results comparing HVab with HVapb RNases, in which replacement of a charged lysine residue with a nonpolar amine produces a dual-specific recognition phenotype.

Dual-Specific *S* Alleles May Play a Key Role in Evolution

We believe that dual-specific alleles may play a pivotal evolutionary role in the generation of new *S* alleles, one of the last conceptual barriers to a full understanding of SI (the other being the identity of the pollen component of the SI system). In essence, the problem of *S* allele generation lies in the contrasting observations that whereas even small populations can contain many different *S* alleles (de Nettancourt, 1997), all attempts to generate *S* alleles with a new specificity by classic mutagenesis have failed and have resulted only in the production of nonfunctional alleles (de Nettancourt, 1977). Clearly, mutations in an *S* allele sequence are more likely to result in breakdown of SI than in a new SI phenotype.

How then do new specificities arise? New S phenotypes have been reported after extensive inbreeding (de Nettancourt, 1977), but unfortunately no molecular information is available about their nature. It has been suggested that the generation of new allelic specificities might proceed by a series of sequential mutagenic steps difficult to monitor because intermediates might require sophisticated analyses for their detection (Lewis, 1951; Fisher, 1961). Indeed, a dual-specificity allele could represent just such an intermediate, and in this study, availability of S_{11} and S_{13} pollen was necessary for its detection. The lack of appropriate genetic systems to detect DSI could thus explain why dual specificities have not been previously observed in SI systems. A further disadvantage is that unlike other systems in which both partners to cell–cell recognition are known, the nature of the pollen component to SI remains elusive.

In our hypothetical model for generating new S phenotypes, we propose that the transition from one allelic specificity to another occurs when stylar and pollen parts evolve step by step (Figure 9). A dual-specific allele is crucial to this scheme because it allows a new recognition specificity to evolve in the plant without it losing its original incompatibility phenotype. Based on both previous work (Matton et al., 1997) and this work, we envisage the following order of three key mutational steps. First, a stylar RNase (S_{11}) with specificity for P_{11} pollen mutates and evolves the capacity (like the dual-specific *HVapb* mutant described here) to recognize and reject a type of pollen (P_{13}) not yet present in the population. The SI system does not break down because the dual-specific allele retains the ability to reject P_{11}. Second, the pollen component P_{11} also evolves by point mutations. The new specificity that will eventually arise (P_{13}) would not be recognizable by S_{11} but does not result in SI breakdown because P_{13}, although phenotypically distinct from P_{11}, is still recognized by the dual-specific allele. Finally, the stylar dual-specific component mutates further to a stage (equivalent to the HVab mutant) at which the recognition of P_{11} is finally lost but the recognition of P_{13} is retained. At this stage, a new *S* allele, consisting of a new male and female pair, has been generated. The intermediate in this process has never lost its SI phenotype, although the specificity of the recognition reaction has been altered.

We do not know the nature of the positive selective pressure that operates to maintain stylar and pollen components as functional pairs, but its effects appear similar to the gene-

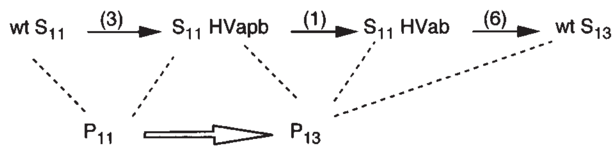

Figure 9. Hypothetical Model of New *S* Allele Generation via Point Mutations.

Diagram depicts the evolution from a wild-type S_{11} RNase to a wild-type S_{13} RNase. The solid arrows represent the amino acid replacements (numbers shown in parentheses) that have either been introduced into the hypervariable regions by experimental site-directed mutagenesis or occurred outside the recognition site. The dotted lines represent recognition of specific tester pollen as experimentally determined by genetic analysis. The open arrow represents hypothetical mutations that must take place within the recognition site of the pollen component. The transitional dual-specific allele (HVapb) contains the maximum number of amino acid changes still allowing recognition of P_{11}. wt, wild type.

for-gene coevolution proposed for host–parasite relationships (Thompson and Burdon, 1992). There is clearly a need to invoke such a positive selective pressure because nonfunctional alleles do not seem to accumulate in natural populations (Campbell and Lawrence, 1981; Richman and Kohn, 1996; see also references in de Nettancourt, 1977). Our results reconcile such a positive selective pressure with the creation of a new and distinct stylar–pollen pair (whose components should not, by definition, recognize the original components from which they have evolved) by providing a means of maintaining a functional SI system while new *S* alleles are generated. Our interpretation suggests that the selective force will be different from inbreeding depression, which would act after mutation in one of the components if this mutation produced a nonfunctional stylar–pollen pair. In our view, it seems advantageous for any positive selective pressure to act before generation of a nonfunctional allele, because nonfunctional alleles would experience no barrier to propagation through the population.

The model as presented here makes no assumptions about the nature of the pollen component to SI. It also makes no predictions concerning the identity or mode of action of the pollen component (because the consequences of a "membrane receptor" or a "cytosolic inhibitor" are irrelevant to the model). The strength of the model lies in the elimination of simultaneity during the production of matching pairs of stylar and pollen components. This is vital given that the probability of simultaneous mutations arising in two different genes is far lower than the probability of a mutation arising in a single gene. Our model, derived from our experimental data on S RNases, places the stylar component as the *DSI* allele that first reaches out toward a new specificity. However, it is equally likely that a similar process could happen in the pollen (i.e., that the pollen component could evolve into a dual-specific allele) because the stylar and pollen parts are genetically linked (i.e., a dual-specific allele in either part must carry its partner through any genetic cross). It will be of great interest to compare the sequences and recognition characteristics of pollen components with the S_{11} and S_{13} alleles when the pollen components of gametophytic SI are at last identified.

METHODS

Plant Material

The diploid (2n = 2x = 24) *Solanum chacoense* self-incompatible genotypes used in this study include line PI458314 (which carries the S_{11} and S_{12} alleles), line PI230582 (which carries the S_{13} and S_{14} alleles), their F_1 hybrid G4 (which carries the S_{12} and S_{14} alleles and which was selected for its high in vitro regenerability [Van Sint Jan et al., 1996]), another F_1 hybrid (which carries the S_{11} and S_{13} alleles), and line PI458316 (which carries the S_{14} and S_{15} alleles). The parental lines were obtained from the Potato Introduction Station (Sturgeon Bay, WI).

Transgenic Plants and Mutagenesis

A HindIII-NcoI fragment (hatched box in Figure 1A) containing the promoter and the 5′ untranslated region of the chitinase gene was ligated to a genomic S_{11} RNase gene engineered to have an NcoI site at the ATG translation initiation codon of the S_{11} preprotein. The S_{11} gene fragment also contains a small (87 bp) intron (black box in Figure 1A) between the hypervariable regions HVa and HVb and 0.8 kb of the 3′ untranslated region. Mutants were obtained through site-directed mutagenesis (Altered Sites II in vitro mutagenesis system; Promega, Madison, WI) using the following mutagenic oligonucleotides (mutated nucleotides are underlined): HVa, 5′-GCCAAAACTTAATTATAAATTTTTCAGTGT-3′; HVb, 5′-GCTTCTGCTCTAAAGGACCAACC-3′; and HVap, 5′-GCCAAAACTTAATTATAACTTTTTCAGTGT-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 polymerase chain reaction amplify the S_{11} 1.7-kb fragment (Pwo DNA polymerase; Roche Diagnostics, Quebec, Canada). The 3.1-kb HindIII-EcoRI chimeras were cloned in the transformation vector pBIN19 (Clontech, Palo Alto, CA) and introduced in *Agrobacterium tumefaciens* LBA4404 by electroporation. Plants were transformed by the leaf disc method using a highly regenerable *S. chacoense* genotype (called G4) carrying the S_{12} and S_{14} alleles (Van Sint Jan et al., 1996). Each construction was sequenced before and after transformation in Agrobacterium to ensure the integrity of the NcoI junction and of the mutated region.

Molecular Analyses

Total RNA was prepared as described (Jones et al., 1985). RNA gel blot analyses (Sambrook et al., 1989) were performed with 10 μg of total RNA, as determined spectrophotometrically, and an S_{13} cDNA probe (Matton et al., 1997). DNA was extracted by following an established procedure (Reiter et al., 1992). DNA gel blot analyses were

performed as described (Sambrook et al., 1989) with 10 μg of genomic DNA per sample digested with EcoRI and probed with the S_{13} cDNA. Because there is only one EcoRI site in the transgene constructs, the number of bands on the DNA gel blot corresponds to the copy number of the transgene. Two-dimensional gel electrophoresis, in-gel RNase activity assays, and tissue prints were performed as described previously (Matton et al., 1997).

Antibodies and Immunochemistry

For protein gel blots, a polyclonal antibody specifically directed against the S_{11} RNase was produced by immunization of a rabbit (Cocalico Biological Inc., Reamstown, PA) with the synthetic peptide KPKLTYNYFSDKMLN (corresponding to the S_{11} RNase HVa region) on a branched multiple antigen peptide (Research Genetics, Huntsville, PA). Protein samples separated by SDS-PAGE and transferred to nitrocellulose were stained with 2% (w/v) Ponceau S to check the uniformity of sample loading. The membrane was then blocked by incubation in Tris-buffered saline containing 1.5% (w/v) BSA (fraction V; Sigma), 2.5% (w/v) instant skim milk powder, and 0.05% (v/v) Tween 80 for 1 hr at room temperature, stained with a 1:1000 dilution of the polyclonal anti-S_{11} antibody for 1 hr at room temperature, rinsed, and visualized with a 1:5000 dilution of a goat anti–rabbit horseradish peroxidase conjugate (Promega) and the Renaissance Western Blot Chemiluminescence Reagent kit (New England Nuclear Life Science Products, Boston, MA), according to the manufacturer's instructions.

Genetic Crosses

The genetic analyses were performed by using pollen from tester stocks with known *S* allele constitution. Crosses were scored as fully compatible when almost every pollination resulted in fruit set, whereas they were considered fully incompatible when pollinations never resulted in fruit formation. Pollen tube growth in the styles was monitored using the aniline blue staining method (Martin, 1959), as described previously (Matton et al., 1997).

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