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Genotype-dependent differences in S_{12}-RNase expression lead to sporadic self-compatibility

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Abstract

Sporadic self-compatibility, the occasional fruit formation after otherwise incompatible pollinations, has been observed in some S_{12}-containing genotypes of *Solanum chacoense* but not in others. We have sequenced this S_{12} allele and analyzed its expression in four different genotypes. The S_{12}-RNase levels were generally less abundant than those of other S-RNases present in the same plants. In addition, two-fold and five-fold differences in the amount of S_{12}-RNase and S_{12} RNA, respectively, were observed among the genotypes analyzed. A comparison with the genetic data showed that genotypes with the highest levels were fully and permanently self-incompatible, whereas those with the lowest levels were those in which sporadic self-compatibility had been observed. The mature protein contains four potential glycosylation sites and genotype-specific differences in the pattern of glycosylation are also observed. Our results suggest the presence of modifier genes which affect, in a genotype-dependent manner, the level of expression and the post-translational modification of the S_{12}-RNase.

Introduction

The past decade has seen considerable progress in understanding self-incompatibility (SI) at a molecular level. SI is a mechanism used by many flowering plant species to prevent self-fertilization and thus promote out-crossing (de Nettancourt, 1977). In the Solanaceae, SI is controlled by an extremely polymorphic *S* locus (de Nettancourt, 1997). Haploid pollen carrying an *S* allele which is also expressed in the style is recognized as self-pollen and growth of these pollen tubes is arrested in the style (Anderson *et al.*, 1989). SI is said to be of the gametophytic type (GSI) in the Solanaceae because it is the genotype of the haploid pollen that determines its SI phenotype.

The only known product of the *S* locus is an S-RNase expressed in the style. A comparison of the S-RNases from many solanaceous species has revealed that they share many common features. All S-RNases are extracellular and contain a hydrophobic N-terminal signal sequence (ca. 22 amino acids long) for ER targeting. All are basic glycoproteins showing a pattern of five conserved regions which contain the amino acid residues required for the RNase activity (Kao and McCubbin, 1996). Indeed, transformation experiments have shown that mutant *S* alleles, in which the RNase activity has been eliminated, are no longer active in pollen rejection (Huang *et al.*, 1994). Lastly, all solanaceous S-RNases contain two hypervariable regions (HVa and HVb), located between the second and third of five conserved regions, which contain important elements for pollen recognition (Matton *et al.*, 1997, 1999). Since the hypervariable regions appear to be a major factor in determining the pollen recognition specificity and because they appear to be located in a part of the molecule different from the RNase activity domain itself (Parry *et al.*, 1998), the pollen recognition domain is also expected to contain hypervariable regions. This idea has precedence

The nucleotide sequence data will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF176533 (cDNA) and AF1911732 (genomic DNA).

in antibody-binding domains (Amit *et al.*, 1986), incompatibility proteins in fungi (Yee and Kronstad, 1953) and plant defense mechanism proteins (Leckie *et al.*, 1999). Interestingly, a very high degree of allelic sequence variability has been found in the recently discovered pollen component to SI in *Brassica* (Schopfer *et al.*, 1999; Takayama *et al.*, 2000).

In spite of the wealth of knowledge concerning this stylar S-RNase, nothing is known about the pollen component to the GSI system, and molecular analyses of the *S* locus now suggest that this genetic locus is much larger and more complex than previously thought (McCubbin and Kao, 1999). Thus, evidence that other genes may modify SI behavior is emerging (Charlesworth *et al.*, 1990; Hosaka and Hanneman, 1998b; Tsukamoto *et al.*, 1999). In particular, studies performed with interspecific hybrids between self-compatible (SC) and SI species have revealed the existence of modifier loci epistatic to the *S* locus (de Nettancourt, 1977 and references herein; Ai *et al.*, 1991; Murfett *et al.*, 1996; Hosaka and Hanneman, 1998b; McClure *et al.*, 1999).

Although in general SI species display a stable SI phenotype, a number of SC variants have been described. A conversion to permanent SC is generally considered to involve mutations in either the stylar or the pollen components to the SI system. As far as the pollen component is concerned, it is well known that polyploids usually display a self-compatible phenotype via the so-called competitive interaction in diploid *S*-heteroallelic pollen whose biochemical bases are still unknown (de Nettancourt, 1977). Other cases of pollen compatibility, whether or not associated with an additional copy of the *S* locus, have also been reported (de Nettancourt, 1977; Thompson *et al.*, 1991; Golz *et al.*, 1999). Considering the stylar components, several examples of SC genotypes that have lost their RNase activity have recently been reported in *Lycopersicon* (Kowyama *et al.*, 1994; Royo *et al.*, 1994), and *Petunia* (Huang *et al.*, 1994; McCubbin *et al.*, 1997), while in some varieties of Japanese pear it is either a deletion of the *S* gene itself that induces SC (Sassa *et al.*, 1997) or a permanent reduction in the production of the S-RNase (Hiratsuka *et al.*, 1999). In addition, blockage of the recognition domain may also result in SC, as illustrated by the relic S-RNase recently described in SC *Nicotiana sylvestris* (Golz *et al.*, 1998) which is glycosylated at a site in the HVa region. Other cases of permanent SC, including pseudo-self-compatibility, i.e. a weakening of the SI reaction leading to variable levels of seed set following selfing, have also been reported. Individuals displaying these types of SC are characterized by qualitative or quantitative defects in products from the *S* locus, possibly caused by modifier loci (Clark *et al.*, 1990; Ai *et al.*, 1991; Hiratsuka *et al.*, 1999; Tsukamoto *et al.*, 1999). In SI *S. chacoense*, the occurrence of SC variants has been reported in the past (Pushkarnath, 1942; Cipar *et al.*, 1964; Xu *et al.*, 1990) and, more recently, an *S*-locus inhibitor gene causing self-compatibility has been described and mapped at the distal end of chromosome 12 (Hosaka and Hanneman, 1998a, b).

Within the framework of our investigations on SI in *S. chacoense*, we have noted that the acquisition of a full SI phenotype by transgenic plants usually correlated with wild-type levels of transgene S-RNase, whereas plants with levels below this threshold were partially compatible (Matton *et al.*, 1997, 1999). We have also previously observed occasional fruit formation after otherwise incompatible pollinations in some genotypes containing a previously uncharacterized S_{12}-RNase (Saba-El-Leil *et al.*, 1994). We have termed this phenomenon sporadic self-compatibility (SSC) as this behavior contrasts with the permanent nature of SC variants described in the literature (see above). We were therefore interested in determining the levels of the naturally occurring S_{12}-RNase in various genotypes to investigate their relationship with the SSC phenotype. Our studies show that the S_{12}-RNase is always poorly expressed when compared to other S-RNases, and its levels are particularly low in genotypes which exhibit SSC. We attribute these genotype-dependent differences to the action of modifier genes unlinked to the *S* locus, and show that they act at both transcriptional and post-translational levels. We suggest that S_{12}-RNase in SSC genotypes is close to the threshold level of RNase required for pollen rejection.

Materials and methods

Plant materials

The parental genotypes PI 458314 (called 314) and PI 230582 (called 582) of *Solanum chacoense* ($2n=2x=24$), containing self-incompatibility alleles $S_{11}S_{12}$ and $S_{13}S_{14}$, respectively, were obtained from the Potato Introduction Station (Sturgeon Bay, WI). Genotype 314 displays sporadic self-compatibility (SSC) whereas genotype 582 is highly

Table 1. S-allele constitution and compatibility behavior of selected *S. chacoense* genotypes.

Genotype	Origin	SI behavior	S alleles
314	Parental	SSC	$S_{11}S_{12}$
582	Parental	SI	$S_{13}S_{14}$
G4	582 × 314	SI	$S_{12}S_{14}$
V22	582 × 314	SI	$S_{11}S_{13}$
V28	582 × 314	SI	$S_{12}S_{13}$
L25	V22 × V28	SSC	$S_{11}S_{12}$
VF60	314 selfed	SI	$S_{12}S_{12}$

self-incompatible (Rivard *et al.,* 1989). Other genotypes investigated here include plant L25 *($S_{11}S_{12}$)*, an F_2 whose parents (V22 and V28 in Table 1) have been previously described as B1 and B2, respectively (Birhman *et al.,* 1994); plant G4 *($S_{12}S_{14}$)*, an F_1 selected for its high regenerability (Van Sint Jan *et al.,* 1996); and plant VF60 ($S_{12}S_{12}$), an individual issued from spontaneous selfing of genotype 314.

The SSC exhibited by genotype 314 was initially observed as fruit formation upon selfings in the greenhouse (Rivard *et al.,* 1989). Furthermore, under field conditions this genotype underwent cycles of spontaneous selfing and produced large numbers of fruit containing numerous seeds (Saba-El-Leil *et al.,* 1994). In both these cases, RFLP analysis of randomly selected plants issued from the seeds set confirmed that they originated by true selfing (Rivard *et al.,* 1994). SSC under greenhouse conditions was also observed for L25.

Genetic crosses were typically monitored by fruit set, and were classified as fully incompatible if they resulted in no fruits set after pollination, and compatible if fruits were set after almost every pollination. Where appropriate, styles stained with aniline blue about 48 h after pollination were examined by fluorescence microscopy to follow pollen tube growth (Matton *et al.,* 1997). Crosses with the S_{12} pollen, obtained from the $S_{12}S_{12}$ homozygote VF60, were classified as incompatible if no pollen tubes were seen at the stylar basis or the ovary, and as compatible if many pollen tubes had entered the ovary.

DNA analysis

Nucleic acids were extracted from styles as described (Jones *et al.,* 1985) and analyzed by standard methods (Sambrook *et al.,* 1989). Hybridization probes were prepared by random priming using a commercial kit (Promega Biotech). A cDNA library from polyadenylated RNA from the styles of genotype L25 was constructed using the cDNA synthesis system of Stratagene (La Jolla, CA) following the manufacturer's instructions. DNA was sequenced using the cycle sequencing system of ABI Prism (Perkin Elmer, NJ) and an ABI automatic sequencer. Sequences were assembled and analyzed with MacVector (Kodak, NJ) and comparisons to GenBank were performed with BLAST (Altschul *et al.,* 1990).

Protein analysis

Proteins were extracted from styles and prepared for either one- or two-dimensional electrophoresis as described (Matton *et al.,* 1997). Proteins stained with Coomassie blue were submitted to the Harvard Microchemistry Facility for microsequencing (Cambridge, MA). Antibodies were raised in rabbits by Cocalico Biologicals (Reamstown, PA) following their standard protocol using a multiple antigen peptide synthesized by Research Genetics (Huntsville, AL). All antibodies were used at a 1:1000 dilution. N-linked glycan side-chains were enzymatically removed by digestion with peptide-N-glycosidase F (PNGase F; Boehringer-Mannheim) according to the manufacturer's instructions. Proteins were purified from stylar extracts by FPLC (Pharmacia) on a Resource S cation exchange column (Pharmacia), equilibrated and washed with 50 mM sodium phosphate pH 6.0 and eluted with a linear gradient of 0–500 mM NaCl in the same buffer at a flow rate of 0.5 ml/min.

Results

Genetic analysis shows a sporadic self-compatibility (SSC) associated with a stylar S_{12} *component*

We have previously reported an RFLP analysis of progeny issued from waves of spontaneous selfing of genotype 314 under field conditions (Rivard *et al.,* 1994). In that study, 9 of the 24 F_1 plants analyzed had $S_{12}S_{12}$ constitution, 15 were $S_{11}S_{12}$ and none were $S_{11}S_{11}$. In a subsequent study, 16 out of 34 plants recovered from seeds after another spontaneous occurrence of self-compatibility in genotype 314 were scored as $S_{12}S_{l2}$ by PCR (K. Hugot, D. Matton, and M. Cappadocia, unpublished observations). Also, when an F_1 progeny issued from the tissue-culture-derived tetraploid genotype L25 (genetic constitution $S_{11}S_{11}S_{12}S_{12}$) crossed with pollen from tetraploid

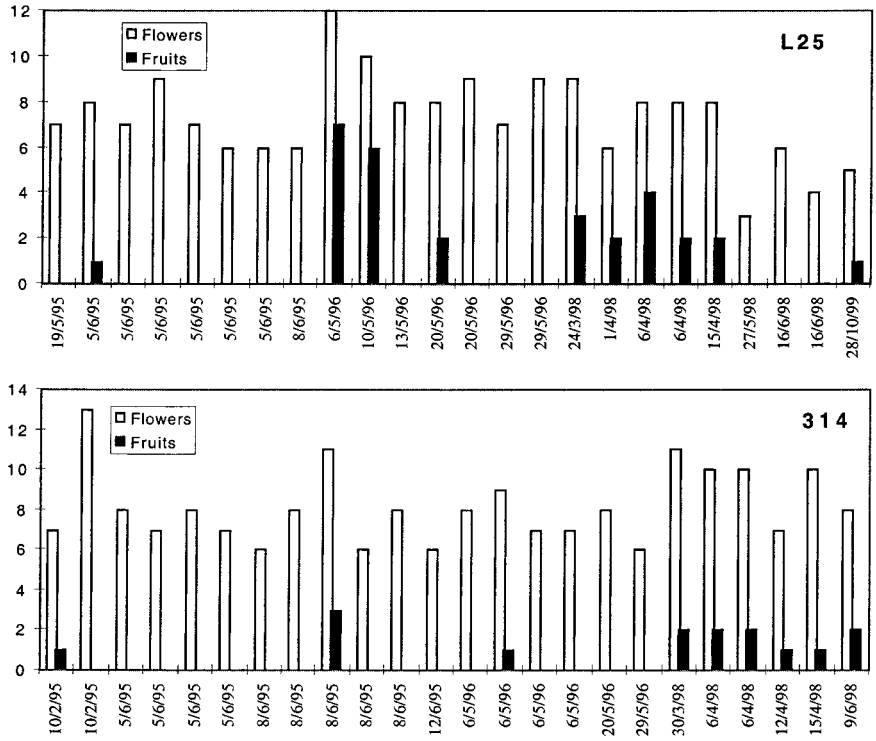

Figure 1. Sporadic self-compatibility of two S_{12}-containing genotypes. $S_{11}S_{12}$-containing plant genotypes L25 and 314 were pollinated under greenhouse conditions. For each plant genotype, pollinations on the day indicated were performed with S_{12}-containing pollen from either genotypes L25, 314 or from the $S_{12}S_{12}$ homozygote VF60. The number of fruit formed (black bars) and the number of flowers pollinated (white bars) are shown for each pollination. Genetic crosses on different dates (dd/mm/yy) were performed with flowers from single individuals.

genotype V28 *($S_{12}S_{12}S_{13}S_{13}$* constitution) was studied, one plant was found to be $S_{12}S_{12}S_{12}S_{12}$, despite the fact that, theoretically, diploid $S_{12}S_{12}$ pollen should have been unable to pass through the style (X. Qin and M. Cappadocia, unpublished). These data suggest that the S_{12} allele is the one involved in the occurrence of SSC.

Sporadic self-compatibility was also observed under greenhouse conditions for genotypes 314 *($S_{11}S_{12}$)* and L25 *($S_{11}S_{12}$)*. Both genotypes occasionally set fruit after self-pollination, reciprocal crosses, or when pollinated with the $S_{12}S_{12}$ genotype VF60. Figure 1 shows a compilation of pollinations, performed at different times over the past few years, with open bars indicating the number of flowers pollinated and closed bars indicating the number of fruit formed. Although these data are derived from crosses performed in the context of other experiments, they illustrate the sporadic nature of the phenomenon. They also reveal an interstyle variation in fruit formation, as fruit formation was typically 20–30% of the flowers pollinated. In contrast, other genotypes containing S_{12} in a different genetic background, such as genotypes G4 *($S_{12}S_{14}$)* or VF60 *($S_{12}S_{12}$)* were found to be strictly self-incompatible after self-pollinations. Since microscopic examination (see later) of pollinated styles from either G4 or VF60 reveals that S_{12} pollen usually stops mid-style and never reaches the lower third of the style, whereas S_{12} pollen tubes could be observed at the stylar basis or even in the ovarian region of genotypes L25 or 324, we conclude that the SSC observed is associated with the stylar S-RNase rather than the pollen component to the S_{12} allele.

Isolation of the S. chacoense S_{12} *cDNA*

A potential S_{12}-RNase was identified by two-dimensional gel electrophoresis analyses as a 31 kDa stylar protein (about 5 kDa larger than the previously described S_{11}- or S_{13}-RNases from *S. chacoense*) cosegregating with the S_{12} allele (Matton *et al.*, 1997). This candidate S_{12}-RNase was isolated from the gel and sequenced. Two of the peptides sequenced were uninformative concerning the identity of the protein, whereas a third (12 amino acids) was identical with

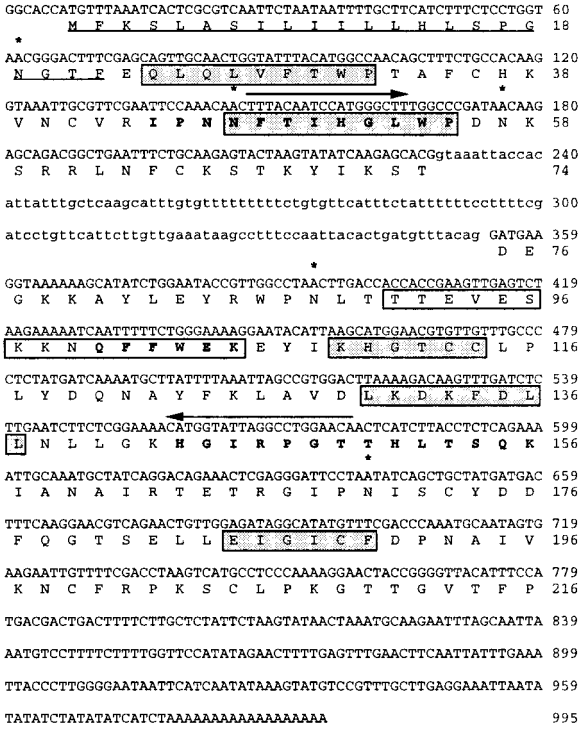

Figure 2. Annotated sequence of a genomic S_{12} DNA. The DNA sequence of the S_{12} gene is shown with the derived protein sequence of the longest open reading frame immediately below. The peptide microsequences (bold) are identical to the derived protein sequence. The five regions conserved in solanaceous S-RNases are shown in shaded boxes, a putative signal peptide of 22 amino acids at the N-terminal is underlined, and putative sites for N-linked glycosylation are marked with asterisks. The region of the protein used for production of the anti-S_{12} (open box) corresponds to the hypervariable region HVb. Arrows indicate the PCR primers initially used to amplify an S_{12}-specific DNA fragment.

the conserved region C2 of other S-RNases. To obtain a cDNA sequence, degenerate oligonucleotides corresponding to all three peptide sequences were synthesized and used to amplify gene fragments from a cDNA library prepared from poly(A) RNA isolated from genotype L25 *($S_{11}S_{12}$).* One pair of oligonucleotides (arrows in Figure 2) amplified a 0.3 kb DNA fragment whose derived protein sequence had 50–60% sequence identity over 100 amino acids to other S-RNases in GenBank ($P<10^{-31}$). This sequence was then used to derive non-degenerate oligonucleotides which were then used for sequencing a full-length S_{12} cDNA. Only a single defined sequence was obtained by amplification of a cDNA library aliquot containing roughly 40 000 clones using both internal and vector oligonucleotides to sequence in both directions. Since the original cDNA library (10^6 independent clones) had been divided into 28 aliquots prior to amplification, and because the 0.3 kb DNA fragment could only be amplified from 11 out of 15 aliquots, the abundance of the S_{12} cDNA is roughly 0.002%. A full-length S_{12} cDNA sequence (Figure 2) was assembled from 5′- and 3′-directed PCR amplifications. The sequence was confirmed by amplifying genomic DNA using oligonucleotides derived from the two furthest ends of the cDNA sequence. The genomic sequences from two different genotypes (L25 and VF60) were identical to each other and to the cDNA sequence, except for the absence of a 126 bp intron located in the hypervariable region HVa (228–354 in Figure 2) of the cDNA.

The derived protein sequence of the cDNA (Figure 2) contains the sequence of the three peptides observed in the protein microsequence (bold), confirming that the cDNA encodes the protein isolated from the two-dimensional gels (100% sequence identity over 32 amino acids). The derived protein sequence also contains the usual five conserved regions (shaded boxes) associated with solanaceous S-RNases, and a strongly hydrophobic N-terminal region of 22 amino acids (underlined) consistent with the presence of an ER-targeting sequence similar to that found in other S-RNases (Singh and Kao, 1992). An unusual feature of the derived protein sequence is the presence of five potential N-linked glycosylation sites (N X S/T; marked with asterisks in Figure 2). The first of these sites is unlikely to be glycosylated in the mature protein since it is located within the signal peptide. The difference between the observed molecular mass on SDS-PAGE (31 kDa) and the predicted molecular mass of the mature protein (23 kDa) suggests that at least some of these sites are glycosylated *in vivo*.

The identity of the cDNA as an authentic S_{12} allele was confirmed by hybridization of an S_{12} probe to both RNA and DNA of plants with different genetic constitution. In all cases the S_{12} cDNA probe hybridized only to nucleic acids isolated from plants containing the S_{12} allele. In DNA gel blots (not shown), VF60 shows the strongest signal, as expected from its $S_{12}S_{12}$ constitution. On RNA gel blots, S_{12}-containing plants show a hybridization signal with an apparent size (0.85 kb) almost identical to the size of the cDNA sequence (Figure 3A). This suggests that the S_{12} RNA has only a small 5′ non-coding region. Taken together, the genetic and molecular data show that our cDNA indeed encodes the *S. chacoense* S_{12}-RNase.

Interestingly, the levels of the S_{12} RNA, based on the amount of hybridization, are different in different plants and appear genotype-dependent. Thus, among

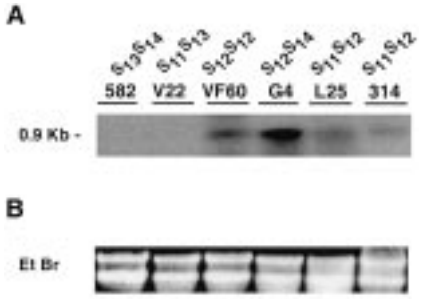

Figure 3. S_{12} cDNA hybridization depends on *S*-allele genotype. A. Stylar RNA samples (10 μg) were electrophoresed on agarose gels in the presence of formaldehyde and transferred to charged nylon filters. The filters were hybridized to an S_{12}-cDNA probe. The genotypes used for the analysis include G4, VF60, L25, 582 and V22. B. Ethidium bromide staining of the rDNA (25S and 18S).

the plants shown in Figure 3A, plants VF60, L25 and 314 have hybridization signals ca. 64±30%, 32±13% and 23±10% of G4, respectively (mean ± SD, $n = 6$).

S_{12}-RNase accumulation is genotype-dependent

To determine the impact of the different S_{12} RNA levels on S_{12}-RNase accumulation, stylar protein extracts were analyzed by protein immunoblots using an anti-S_{12} antibody raised against the peptide TTEVESKKNQFFWEK (open box in Figure 2). In most cases, the antibody cross-reacted with two proteins with an apparent molecular mass of 29 and 31 kDa whose relative amounts were found to vary in different genotypes (Figure 4A). In VF60, all detectable immunoreactive protein is found at 31 kDa, whereas in other genotypes, such as 314 and L25, up to half of the immunoreactive protein is found at 29 kDa. These two molecular mass bands represent different glycosylated forms of the same protein, because only a single protein is observed after deglycosylation with PNGase F (Figure 4C).

Densitometric scans, averaged from five independent experiments, indicate that S_{12}-RNase levels in VF60, L25 and 314 are 69±15%, 44±8% and 47±18% of the levels found in G4, respectively. These values follow the same trend as the RNA gel blot results, and suggest that SSC in L25 and 314, might be related to their lower S_{12}-RNase content. To assess the impact of the different RNase levels on pollen tube growth, S_{12} pollen from VF60 growing in styles of different genotypes was examined by UV microscopy. These observations revealed that the genotypes containing high levels of immunoreactive protein such as

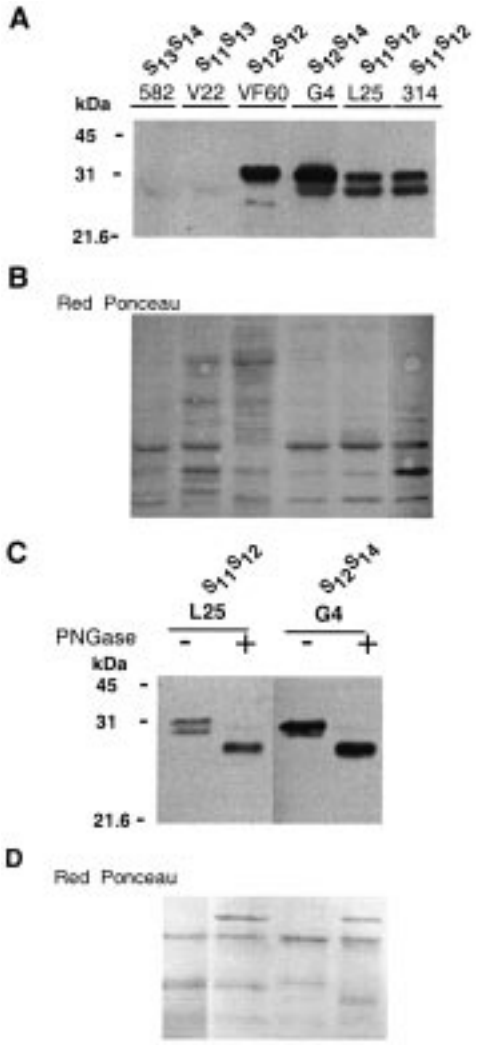

Figure 4. Styles of different genotypes contain different levels of two glycosylated S_{12}-RNases. Stylar protein samples (10 μg) were electrophoresed on acrylamide gels, transferred electrophoretically to nitrocellulose and challenged with an anti-S_{12}, with the position of antibody binding visualized by chemiluminescence. A. Protein samples from plants of different genotypes contain differing amounts of two proteins (29 kDa and 31 kDa apparent molecular mass) which cross-react with the antibody. B. Ponceau red stain of the membrane shown in A. C. Protein samples from plants L25 and G4 with or without deglycolysation using PNGase. D. Ponceau red stain of the membrane shown in C.

VF60 or G4 were those where no S_{12} pollen tubes reach the stylar base (Figure 5A). In contrast, in L25 and 314, where lower amounts of immunoreactive protein are observed, a few pollen tubes occasionally enter the ovarian region of some styles (Figure 5B, C), consistent with the style-to-style variation observed in breeding behavior (Figure 1). Thus, SSC appears

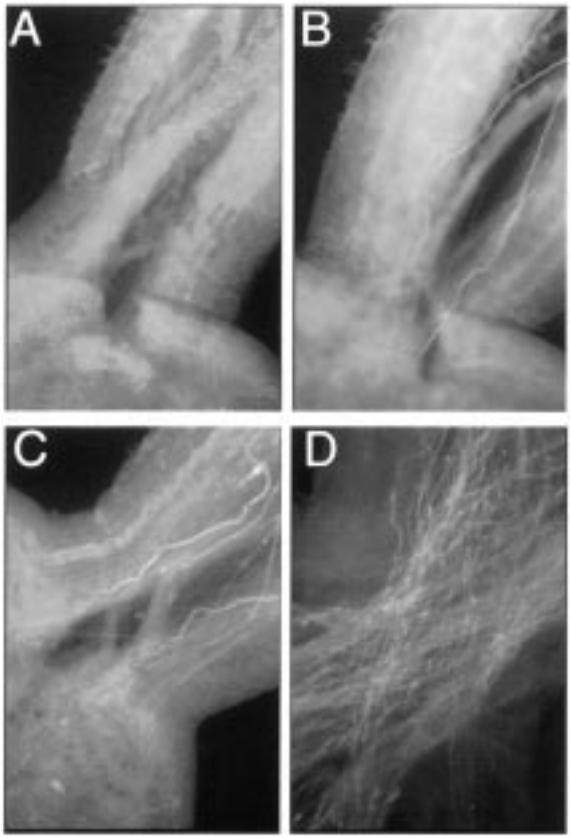

Figure 5. S_{12} pollen tubes can reach the ovarian region in plants displaying SSC. Squashes of stylar bases, stained with aniline blue 48 h after pollination with pollen from an $S_{12}S_{12}$ individual. A. G4 styles, an incompatible pollination with no pollen tubes at the stylar base. B, C. 314 (B) and L25 (C) styles, with some pollen tubes at the ovarian region. D. 582 styles, a compatible pollination with numerous pollen tubes at the stylar base.

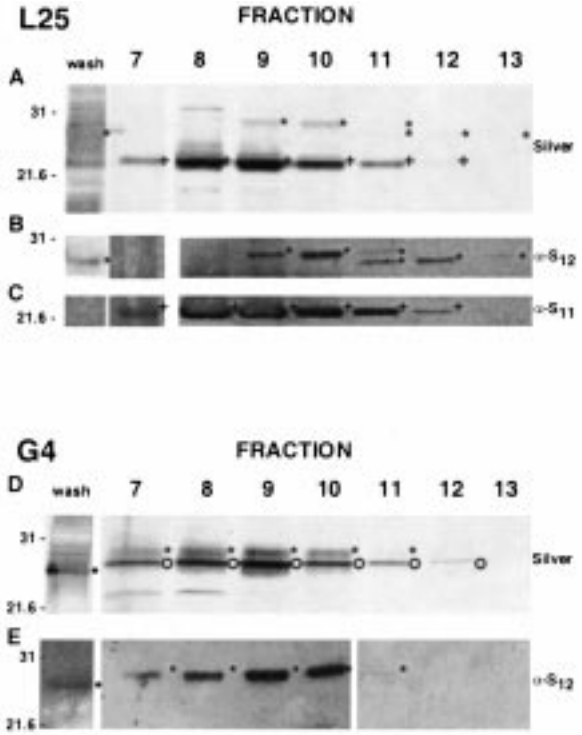

Figure 6. Differential glycosylation of S_{12}-RNase. Stylar protein samples (100 μg), the S_{11}/S_{12} genotype L25 (A, B, C) or the $S_{12}S_{14}$ genotype G4 (D, E) were loaded onto a cation exchange column, washed, and eluted with salt. The wash and different fractions of the eluate were electrophoresed on acrylamide gels and either stained with silver (A, D) or transferred electrophoretically onto nitrocellulose and challenged with an anti-S_{12} (B, E) or an anti-S_{11} (C). All fractions which reacted with the anti-S_{12} antibody are shown.

to correlate with a diminished capacity of S_{12}-RNase containing styles to arrest S_{12} pollen tube growth.

We were also curious as to why our two-dimensional gel analysis of basic stylar proteins in G4 plants (Matton *et al.*, 1997) identified only a single S_{12} isoform, while the anti-S_{12}-RNase immunoblots showed two different size classes (Figure 4). To address this question, protein extracts from L25 and G4 were purified by cation exchange chromatography and the anti-S_{12} reactivity measured in the different column fractions (asterisks in Figure 6A, B). In extracts from L25 styles, two S_{12}-RNase glycoforms, with a molecular mass of 31 and 29 kDa, eluted maximally in fractions 10 and 12, respectively. Surprisingly, an additional 29 kDa glycoform was not retained by the cation exchange column and appeared in the flowthrough during column loading (wash fraction in Figure 6B). Thus L25 contains three different glycoforms, and, despite the fact that one isoform seems acidic, all isoforms were active when tested using in-gel RNase assays (data not shown). The presence of the S_{12}-RNase in the column wash was not due to column overload because the S_{11}-RNase, identified by reaction with an anti-S_{11} antibody, was never observed in the wash fraction (Figure 6C). In extracts from G4 styles, only two S_{12} isoforms were present, the 31 kDa form eluting principally in fraction 10 and the 29 kDa form not retained by the column (asterisks in Figure 6D, E). The presence of an acidic S_{12}-RNase in these extracts thus explains why only a single S_{12}-RNase band was identified by our previous two-dimensional gel analyses of basic stylar proteins in G4. Furthermore, the presence of a different number of S_{12} glycoforms in L25 and G4, as well as the absence of the 29 kDa band in VF60, clearly shows that

the differences in the glycosylation of the S_{12}-RNase are genotype-dependent.

The partial purification of the S-RNases by ion exchange chromatography also allows a comparison of the amounts of the different RNases within each genotype. Silver staining of the different column fractions shows that the levels of S_{12}-RNase are much lower than those of the S_{11}-RNase in L25 extracts (compare asterisks with crosses in Figure 6A) and much lower than the S_{14}-RNase in G4 extracts (compare asterisks with open circles in Figure 6D). Thus it appears that the general level of S_{12}-RNase accumulation is lower than that of other S-RNases.

Discussion

We report here the sequence of the *S_{12}* incompatibility allele of *S. chacoense*. The sequence is similar to other known solanaceous S-RNase sequences, and contains those conserved amino acids required for RNase activity as well as the pattern of five constant and two hypervariable regions typical for these proteins. The mature protein contains multiple potential glycosylation sites, a situation found only in few other S-RNases (Woodward *et al.*, 1989; Oxley *et al.*, 1996). The evidence that the cDNA sequenced corresponds to the *S_{12}* allele is based on the 100% sequence identity with the protein microsequence data and the cosegregation of the protein with the *S_{12}* SI phenotype. In addition, the RNA gel blot analysis using the cDNA sequence as a probe also shows cosegregation of the *S_{12}* allele sequence with the *S_{12}* phenotype (Figure 3). We conclude from this that the cDNA isolated represents the sequence of the S_{12}-RNase.

We have found that expression of the S_{12}-RNase is affected by the plant genotype, possibly through the action of a modifier locus. *S_{12}* RNA accumulation is different in the different genotypes as shown by RNA gel blots (Figure 3A). These differences are mirrored by differences in the amount of S_{12}-RNase accumulation as measured by protein immunoblots (Figure 4). Changes in the amount of antibody signal observed between the different genotypes is not due to changes in glycosylation, as no increase in signal is observed after deglycosylation. Therefore, the differences in S_{12}-RNase appear to be directly related to changes in *S_{12}* RNA, and may reflect differences in transcription rates, in turnover rates, or a combination of the two. It would thus be of interest to compare the sequence of the *S_{12}* gene promoter with that of the *S_{11}* gene.

In *Brassica campestris*, a sharp decrease in SLG expression leading to self-compatibility has been attributed to a recessive mutation at the modifier locus SCF1 (Nasrallah *et al.*, 1992), although the implication of SLG in the *Brassica* SI reaction has been questioned (Gaude *et al.*, 1995). The recent isolation of the HT gene in *N. alata* also supports the existence of modifier gene(s) unlinked to the *S* locus yet involved in SI. In these experiments, interspecific hybrids expressing antisense HT were SC, although the reduced levels of HT were not found to influence the S-RNase levels per se (McClure *et al.*, 1999). Studies on interspecific hybrids between SC *P. hybrida* and SI *P. inflata* have also revealed that the function of the *S_x* allele depended on the genetic background; indeed, F_1 hybrid progeny contained SC and SI individuals in a ca. 1:1 ratio (Ai *et al.*, 1991). More recently, segregation studies on interspecific hybrids between *S. chacoense* and *S. phureja* have revealed an *S*-locus inhibitor gene with sporophytic action (Hosaka and Hanneman, 1998a, b). If we now consider intraspecific variations in SI phenotype, these have recently been described in *P. axillaris* (Tsukamoto *et al.*, 1999). In that study, 30 plants carrying and expressing the S_{13}-RNase were SI, while three others, carrying but not expressing the same allele, were SC. Since both SI and SC plants had originated from a single population, the authors rightly suspected the action of a modifier locus specifically affecting *S_{13}* allele expression.

In addition to differences in the level of S_{12}-RNase expression, we have observed that this enzyme is also subject to genotype-specific differences in glycosylation. For example, untreated extracts from G4 and VF60 have a different number of bands cross-reacting with the antibody, and these differences disappear after treatment with the PNGase F. Based on the average size of sugar groups in *N. alata* (0.9–1.9 kDa; Oxley *et al.*, 1996) and the sizes of the 29 kDa and 31 kDa bands relative to the predicted molecular mass of the sample (23 kDa), one interpretation of these two bands is that either three or four of the potential glycosylation sites are occupied. Alternatively, it is possible that the two size classes of glycosylated S_{12}-RNase may be due to differential modification of the sugar groups at each of the four potential sites. Modifications of sugar groups in *N. alata* S-RNase have previously been observed and described as microheterogeneous (Oxley *et al.*, 1996). We do not know if this microheterogeneity is present in our S_{12}-RNase since we are comparing different genotypes rather than documenting the number of different sugar groups which can be found in

the S-RNase of a given genotype. However, it is clear that modification of sugar groups must occur in *S. chacoense*, as shown by the different isoelectric points of the 29 kDa isoforms found in L25 extracts. We conclude that there are post-translational modifications to the glycosylation pattern of the S_{12}-RNase and that these differences may involve modification of the type and/or number of sugar groups. The dependence on the genetic background of the plants suggests, once again, that these modifications reflect the action of modifier genes. Although glycan-processing enzymes have been proposed to be part of the *S* locus in *N. alata* (Oxley *et al.*, 1996), the observation that the number of S_{12}-RNase glycoforms detected in L25 (3 isoforms; Figure 6) is greater than in G4 (2 isoforms; Figure 6) suggests that the genes encoding these enzymes may lie outside the *S* locus in *S. chacoense*.

The occurrence of SSC in two different plant genotypes and not in others shows that the genetic background of the plant can influence the pollen rejection phenotype of the S_{12} allele. Modification of the genetic background, especially through interspecific hybridization, is known to sometimes cause breakdown of SI and to result in variable strength and persistence of SC (de Nettancourt, 1977). In those cases where genetic analyses of the SC plants have been conducted, the breakdown of SI could be attributed to one or more modifier genes unlinked to the *S* locus. This in turn has led to the idea of a possible polygenic control of SI and its dependence upon a certain degree of heterozygosity (de Nettancourt, 1977). Further genetic analyses of our material should reveal if the modifier genes implicated in expression of the S_{12}-RNase are also unlinked to the *S*-locus. It is also worth noting that some episodes of SSC in both L25 and 314 occurred on similar dates, which suggests that environmental and/or physiological factors may have played a role in this phenomenon.

Why then is the SSC restricted to only some genotypes carrying the S_{12} allele? First of all, we observe that S_{12}-RNase levels appear to be generally low, at least compared to the S_{11}- and S_{14}-RNases in L25 and G4, respectively (Figure 6). Second, in some S_{12} genotypes, such as 314 or L25, S_{12}-RNase accumulation is particularly low (Figure 4). If these low S_{12}-RNase levels were just above the threshold required to fully reject S_{12} pollen, then physiological or environmental factors could sometimes induce a transient breakdown of SI by further reducing the S_{12}-RNase level. In this regard, the findings with the endogenous S_{12}-RNase described here are different from those reported in *P. axillaris* where the occurrence of SC was permanent in three individuals and was caused by their inability to produce any detectable amount of the S_{13}-RNase. They are also different from the permanent SC reported in the Osa-Nijisseiki Japanese pear mutant, where the S_4-RNase was present at one-third the levels found in the self-incompatible Nijisseiki cultivar (Hiratsuka *et al.*, 1999). The SSC phenotype is not permanent and, curiously, when present, is not manifested by all styles of the same plant. This additional unusual feature documents a naturally occurring interstyle variation not detectable with other, more highly expressed, S-RNases. Indeed, interstyle variation has been previously observed in transgenic plants but only when they express low levels of S-RNase (Matton *et al.*, 1997, 1999). Detailed genetic analysis of these transgenic plants revealed that different styles from the same plant could have a different pollen rejection phenotype. This phenotype, defined as partially compatible, was found only in plants with reduced levels of transgene expression. Style-by-style analyses might help to define more precisely the S_{12}-RNase threshold required for full rejection of S_{12} pollen. Further genetic analyses, conducted on progeny obtained from L25 and 314, may also allow a more precise evaluation of the S_{12}-RNase threshold and may, in addition, shed some light on the nature of the modifier genes involved in modulating SI.

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