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RESEARCH PAPER

Style-by-style analysis of two sporadic self-compatible *Solanum chacoense* lines supports a primary role for S-RNases in determining pollen rejection thresholds

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Abstract

A method for the quantification of S-RNase levels in single styles of self-incompatible *Solanum chacoense* was developed and applied toward an experimental determination of the S-RNase threshold required for pollen rejection. It was found that, when single style values are averaged, accumulated levels of the S_{11}- and S_{12}-RNases can differ up to 10-fold within a genotype, while accumulated levels of the S_{12}-RNase can differ by over 3-fold when different genotypes are compared. Surprisingly, the amount of S_{12}-RNase accumulated in different styles of the same plant can differ by over 20-fold. A low level of 160 ng S-RNase in individual styles of fully incompatible plants, and a high value of 68 ng in a sporadic self-compatible (SSC) line during a bout of complete compatibility was measured, suggesting that these values bracket the threshold level of S-RNase needed for pollen rejection. Remarkably, correlations of S-RNase values to average fruit sets in different plant lines displaying sporadic self-compatibility (SSC) to different extents as well as to fruit set in immature flowers, are all consistent with a threshold value of 80 ng S_{12}-RNase. Taken together, these results suggest that S-RNase levels alone are the principal determinant of the incompatibility phenotype. Interestingly, while the S-RNase threshold required for rejection of S_{12}-pollen from a given genetic background is the same in styles of different genetic backgrounds, it is different when pollen donors of different genetic backgrounds are used. These results reveal a previously unsuspected level of complexity in the incompatibility reaction.

Key words: Gametophytic self-incompatibility, single style analysis, S-RNase, *Solanum chacoense*, threshold.

Introduction

Self-incompatibility (SI) is a widespread genetic mechanism used by many species of flowering plants to prevent inbreeding by promoting outcrossing. This prezygotic barrier is based on recognition of the gene products expressed in specialized cells of the pistil by those expressed in the pollen, which results in rejection of self- but acceptance of non-self pollen (de Nettancourt, 1977, 2001). The Solanaceae, Rosaceae, and Scrophulariaceae are characterized by gametophytic SI, or GSI, where the incompatibility phenotype of the haploid pollen is determined by its own genotype. In these families the male and female determinants to SI are both encoded at a highly complex and multiallelic *S*-locus. Pollen rejection occurs when the S-haplotype of the haploid pollen matches either of the two S-haplotypes of the diploid pistil, and it takes place inside the upper part of the style. The pistillar gene product to SI is a highly polymorphic ribonuclease termed S-RNase (McClure *et al.*, 1989) that is synthesized by the cells of the transmitting tissue of the style and secreted into the surrounding extracellular matrix where the pollen tubes grow. The pollen determinant to SI (pollen-S gene product) has recently been identified as a polymorphic F-box protein, termed either SLF (S-locus F-box) or SFB (for S-haplotype-specific F-box) by the various authors (for details see Kao and Tsukamoto, 2004; McClure, 2004).

The cytotoxic action of the S-RNases mediates rejection of incompatible pollen by degrading pollen tube RNA in an S-haplotype-specific manner, although the minimal amount of S-RNase required for pollen rejection has not been

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determined. S-RNases have been shown to enter and accumulate inside the pollen tubes in a haplotype-independent manner (Luu *et al.*, 2000), suggesting that the pollen contains proteins able to inhibit or destroy S-RNases. The mechanism whereby S-RNases penetrate inside the pollen tubes, however, is unknown. It has been suggested that this may occur either by endocytosis, via inclusion into a membrane-bound compartment (McClure, 2004) or through a receptor (or a receptor complex) that recognizes a conserved domain of the S-RNase (Kao and Tsukamoto, 2004). In this regard, the involvement of one of the most attractive possibilities (the conserved C4 region in S-RNases) in uptake has recently been ruled out (Qin *et al.*, 2005).

Permanent self-compatibility (SC) has been reported several times among SI species (for a review, see de Nettancourt, 1977). In most cases, it can be attributed to mutations directly affecting either the pistillar or the pollen determinants to SI (de Nettancourt, 2001). Examples of the former include mutations at the S-RNase gene causing loss of the RNase activity as reported in *Lycopersicon* (Kowyama *et al.*, 1994; Royo *et al.*, 1994) and *Petunia* (Huang *et al.*, 1994; McCubbin *et al.*, 1997), or deletion of the S-RNase gene itself (Sassa *et al.*, 1997). With regard to self-compatibility resulting from pollen-part mutations, it is most often associated with the so-called competition effect that takes place when two distinct pollen-S genes are expressed in the same pollen grain. An extra pollen-S gene introduced into a host plant by transgenesis (Qiao *et al.*, 2004; Sijacic *et al.*, 2004) produces the same effect. In some instances, pollen compatibility has been shown to result from the loss of pollen function (Tsukamoto *et al.*, 2003), or from mutations affecting the pollen S-gene (Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005), or from deletion of the pollen S-gene itself (Sonneveld *et al.*, 2005). This last case is particularly important as it suggests S-RNases are inactive in pollen tubes without their cognate pollen-S, as predicted by the two-component inhibitor model (Luu *et al.*, 2000, 2001). Lastly, other cases of SC have been shown to depend on so-called modifier genes, located outside the S-locus, that appear to be required for proper manifestation of the SI response, such as HT-B (O'Brien *et al.*, 2002) or a stylar 120 kDa glycoprotein in *Nicotiana* (Hancock *et al.*, 2005) (for a further discussion see Kao and Tsukamoto, 2004).

A special category of partial incompatibility is represented by pseudo-self-compatibility (i.e. formation of fruits containing variable amounts of seeds observed after crosses expected to be incompatible) (Clark *et al.*, 1990) and sporadic self-compatibility (i.e. occasional fruit formation after crosses expected to be incompatible) (de Nettancourt *et al.*, 1971; Qin *et al.*, 2001). In particular, sporadic self-compatibility has been observed in some but not all S_{12}-containing genotypes of *Solanum chacoense*, and is characterized by occasional bouts of self-compatibility with S_{12} pollen that can affect from 10% to 60% of the styles on a given plant. Expression of the S_{12} allele has been analysed in several plant lines and genotype-specific differences were found in the amount of S_{12}-RNase and S_{12}-mRNA. As sporadic self-compatibility occurred only in those genotypes with the lowest average S_{12}-RNase levels (Qin *et al.*, 2001), it is proposed that there may be a variation between flowers that could result in levels of S-RNase in some individual styles too low to reject otherwise incompatible pollen. Indeed, style-to-style variations in S-RNase levels could explain both pseudo and sporadic compatibility but has not previously been demonstrated.

Weakening of the SI response, associated with a reduced level of S-RNases present in the pistil, has led to the hypothesis that a threshold level of the RNase is required for full expression of the SI phenotype (Clark *et al.*, 1990). Support for this idea has been provided by studies on partially compatible Japanese pear cultivars displaying low levels of S-RNase expression (Hiratsuka *et al.*, 1999, 2001; Zhang and Hiratsuka, 2000; Hiratsuka and Zhang, 2002), and by the partial incompatibility of plants expressing an S-RNase transgene at levels significantly below those produced by the endogenous alleles (Lee *et al.*, 1994; Murfett *et al.*, 1994; Matton *et al.*, 1997, 1999; Qin *et al.*, 2005). Finally, accumulation of the S-RNases in the style during flower development is temporally regulated and the increase in S-RNase levels correlates with the acquisition of the incompatibility phenotype (Xu *et al.*, 1990; Clark *et al.*, 1990; Zhang and Hiratsuka, 2000). All these examples are consistent with the hypothesis that a threshold level of S-RNase is required to inhibit the growth of incompatible pollen tubes. However, the threshold itself has never been measured, and the factors that potentially influence it (S-RNase haplotype, pollen genotype, environmental conditions) have not been assessed.

The aim of the present study was the experimental determination of an S-RNase threshold and a preliminary evaluation of the factors that may influence it. To do so, advantage was taken of the sporadic self-compatible phenotype of some of our S_{12}-RNase containing plant lines (Qin *et al.*, 2001). A technique was developed for measurement of the S-RNase levels in single styles, and a definite S-RNase threshold for a particular pollen haplotype in a particular genetic background was found. However, it was also found that the S-RNase threshold required for rejection of this pollen haplotype can vary depending on the genetic background of the pollen donor. It was also noted that the S-RNase threshold differs when incompatibility is defined either by fruit formation or by the lack of pollen tubes entering the ovarian region.

Materials and methods

Plant genotypes

The plant material used in these experiments includes the fully self-incompatible G4 (S_{12} S_{14}), VF60 ($S_{12}S_{12}$), 582 ($S_{13}S_{14}$) genetic lines,

as well as the two sporadically self-compatible L25 ($S_{11}S_{12}$) and 314 ($S_{11}S_{12}$) lines of *Solanum chacoense* ($2n=2x=24$) described previously (Qin *et al.*, 2001). In addition, an individual called TP48 ($S_{12}S_{12}$) issued from the selfed 314 line (Qin *et al.*, 2001), and a plant named 2548 ($S_{12}S_{12}$) produced by crossing L25 as pistillate parent with TP48 as staminate parent, and selected for its high vigour, pollen fertility, and high *in vitro* regenerability, were also used.

Genetic crosses

Genetic crosses on recently open flowers were always made with fresh pollen collected from plants of known S-haplotype constitution grown in the Montreal Botanical Garden greenhouses at 23±2 °C under natural light conditions. Pollen viability was estimated by staining with aceto-carmine. Bud pollinations were performed on flower buds at 3, 2, and 1 d before anthesis (DBA). Crosses were classified as fully incompatible if there was no fruit formation after pollination, and compatible when fruits were formed after almost every pollination. Where appropriate, pollen tube growth was monitored by staining the styles with aniline blue about 48 h after pollination, followed by observations by fluorescence microscopy as previously described (Matton *et al.*, 1997). In some cases, the styles were observed 72 h after pollination. In other cases, flowers were gently shaken (touched) 4–5 d post-pollination, and the styles of fallen flowers were observed by fluorescence microscopy to determine if the lack of fruit set could be attributed to self-incompatibility.

Progeny analysis

Seeds obtained from bud pollination of the L25 line selfed at 3 DBA were germinated *in vitro* as described previously (Van Sint Jan *et al.*, 1996), and the S-constitution of the resulting plantlets assessed by PCR. For each genotype, five leaf discs of 2 mm diameter were crushed with a plastic mortar in 20 µl 0.25 N NaOH, and incubated for 5 min at 95 °C. The mixture was then neutralized with 20 µl 0.25 N HCl, 20 µl TRIS-HCl pH 8.0 and 0.5% w/v Igepal CA-630 (Sigma). The tubes were incubated for five additional minutes at 95 °C, centrifuged for 1 min at 5000 rpm, the supernatant collected, and immediately used for the PCR reactions (40 cycles of 94 °C 30 s, 55 °C 30 s, and 70 °C 1 min) using a commercial PCR buffer (Promega) and *Taq* polymerase (Promega). The primers used for analysis of the S_{11} allele were 5′-CTATTTCAGTGTAAGCAGC-3′ and 5′-ATTTCTAGAGGACGAAAAAATATTTTC-3′, while primers 5′-TAACTTGACCACCACCG-3′ and 5′-GTCATGGAAATGTAACCC-3′ were used for the S_{12} allele.

Expression of S_{11}- and S_{12}-RNases in E. coli

The cDNA clones encoding S_{11}- and S_{12}-RNases were first mutated at the active site to avoid possible RNase activity toxic to the *E. coli* host cells, then cloned into an expression vector pQE30 (Qiagen, Valencia, CA). For the S_{11}-RNase, the histidine encoded by CAC in the conserved region C2 was substituted with arginine (CGT). The three primers used for site-directed mutagenesis were S_{11}His*Sma*I (at the C-terminal end of the coding sequence), 5′-CTCTCTCTCTCCCCGGGCAAGGACGAAAAAATATTTCC-3′; S_{11}His*Sac*I (corresponding to the N-terminal end of the mature coding sequence), 5′-GAGAGAGAGAGAGCTCAAATTGCAACTGGTATTA-3′; and S_{11}cmc2 (containing the substituted codon), 5′-CCTTATCCGGCCAAAGACC**ACG**AATCGTAAAGTTTTTTG-3′. For the S_{12}-RNase, the histidine encoded by CAT in the C2 conserved region was substituted with arginine (CGT). Two pairs of primers, S_{12} *Hin*dIII (at the C-terminal end of the coding sequence), 5′-CTCTCTCTCTAAGCTTGGAAATGTAACCCCGGTA-3′; MuS_{12}-HisA, 5′-AACTTTACAATC**CGT**GGGCTTTGGCCC-3′ and S_{12} *Bam*HI (corresponding to the N-terminal end of the mature coding sequence), 5′-GAGAGAGAGAGGATCCGAGCAGTTGCAACTGGT-3′, MuS_{12}-HisB, 5′-GGGCCAAAGCCC**ACG**GATTGTAAAGTT-3′ were used to amplify the mutant clone. The mutated cDNA fragments were cloned into pQE30 between the *Sma*I and *Sac*I sites for S_{11} and between the *Bam*HI and *Hin*dIII sites for S_{12}. The sequences of both mutated clones were confirmed by sequencing and named pQE30-S11-hisΔ-C2 and pQE30-S12-hisΔ-C2.

These two plasmids were transformed separately into competent M15 cells, and protein expression was induced by adding IPTG to a final concentration of 1 mM. The cultures were typically grown for 4–5 h before the cells were harvested. The target proteins were purified with Ni-NTA resin (Qiagen, Maryland, USA), electrophoresed on SDS-PAGE, then eluted from gel slices using an Electro-Elutor (Bio-Rad, California, USA) following the manufacturer's protocol. The purity of both proteins was confirmed by Coomassie blue staining after SDS-PAGE and western blot analysis.

Quantification of the standard S_{11} and S_{12} proteins

The concentration of purified S_{11} and S_{12} proteins was determined both by OD_{280} and by the Micro BCA Protein Assay Kit (Pierce Inc., Illinois, USA) following the manufacture's protocol. The two measures gave similar results and were thus used to calibrate S-RNases measurements in styles of *S. chacoense*. One batch of each purified protein was used as the standard for all gels.

Western blots and quantitative analysis of S_{11} and S_{12} RNases in individual styles

For western blots, plant styles were collected and frozen immediately in liquid nitrogen. The term 'style' as used here comprises both the style *per se* as well as the stigmatic region. Proteins were extracted from individual styles using 50 µl extraction buffer (0.05M TRIS pH 8.5, 1 mM DTT, 1 mM EDTA, 0.05 M $CaCl_2$, 1 mM PMSF). Typically, 5× SDS sample buffer was added to 25 µl crude extract from each individual style and electrophoresed on SDS-PAGE. Immunological detection of the S-RNases is linear over the range used, so only one aliquot containing 400 ng of each purified S-RNase was run on each gel for standardization. The proteins were transferred to nitrocellulose membranes and stained with 2 (w/v) Ponceau red as a control for a uniform protein load; S-RNase measurements from samples with visibly different protein loads were excluded from these analyses. The membranes were blocked by an overnight incubation with TBS-T (TRIS-buffered saline containing 1.5% w/v BSA fraction V (Sigma) and 0 w/v Tween 80), incubated for 2 h at room temperature with a 1:1000 dilution of either polyclonal anti-S_{11} (Matton *et al.*, 1999) or anti-S_{12} (Qin *et al.*, 2001) antibodies, rinsed with TBS-T three times, and incubated with 4 µl of 0.5 µCi mmol^{-1} I^{125}-protein A (Perkin-Elmer) in 5 ml TBS-T for 2 h. After washing three times with TBS-T, the membranes were exposed with a Phosphor screen for 12–76 h at room temperature and the screen imaged using a PhosphorImager scanner (Amersham Bioscience). The data on the scanned images were quantified using the software supplied by the manufacturer. Both antibodies used are specific for their respective substrates.

Results

S-RNase levels can be reproducibly assayed in single styles

The threshold hypothesis for S-RNase-mediated pollen rejection posits that an incompatible phenotype requires a minimum level of S-RNase within the styles. To determine this level experimentally, it was first necessary to

develop a technique that would permit the absolute levels of S-RNase to be accurately and reproducibly measured in extracts from single styles. This assay thus requires two elements, a sensitive detection system and a calibration method to calculate the amount of S-RNase at ng level. For the latter, S-RNase protein standards were prepared by expressing an inactive S-RNase as a His-tagged construct in bacteria. Two tagged constructs, an S_{11}-RNase and an S_{12}-RNase, were purified by Ni-affinity chromatography and SDS-PAGE elution, and were homogenous by the criteria of Coomassie blue staining (Fig. 1).

For development of a sensitive and easily quantifiable assay, western blotting was coupled using an I^{125}-labelled protein A with detection using a PhosphorImager. To characterize the response to the antibodies, standard curves using different amounts of both S-RNases were prepared. The immunological response is linear under these conditions (Fig. 1C). To characterize the precision of the measurements, S_{11}-RNase levels in each of four equal aliquots of an individual style extract were compared (Fig. 2). These S_{11}-RNase measurements have a coefficient of variation of only 5% and show that the method can faithfully assess the RNase levels in individual styles.

Since there was interest in measuring levels of both S_{11}- and S_{12}-RNases in single style extracts, tests were carried out to see if single transfers could be analysed using both anti-S_{11}- and anti-S_{12}-RNases. In one experiment, two membranes containing half the extracts from 18 styles of plant 314 ($S_{11}S_{12}$ genotype) were prepared. One membrane was treated first with the anti-S_{11}-RNase and the S-RNase levels quantitated using the PhosphorImager. This membrane was then stripped and treated with the anti-S_{12}-RNase (Fig. 3A). A second membrane, containing the other half of the same samples was similarly treated except that the order of the two antibodies on the membrane was reversed (Fig. 3B). An additional 18 styles from the plant L25 (also $S_{11}S_{12}$) were analysed in parallel using the same protocol (Fig. 3C, D). All samples show a substantial decrease in calculated S_{11}-RNase (Fig. 3E) and S_{12}-RNase (Fig. 3F) levels when measured on previously used membranes. It was concluded that this technique does not accommodate multiple analyses from the same membrane. However, as reliable measurements were obtained from one-half of the stylar extracts, both S-RNases can be measured in a single style extract by simply preparing two membranes from each sample. It was also noted from this preliminary study

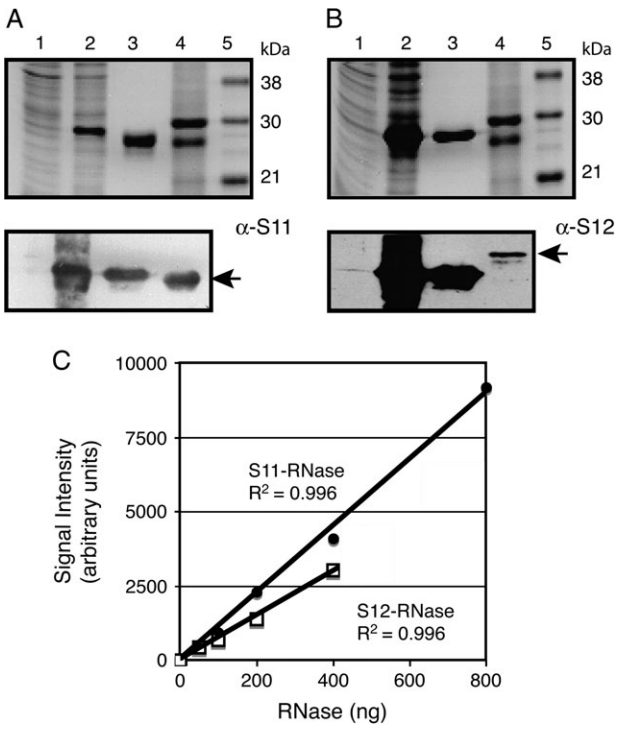

Fig. 1. S_{11} and S_{12}-RNase standards are pure by Coomassie blue staining. (A, B) Bacterial cells transformed with either an S_{11}-RNase (A) or an S_{12}-RNase (B) cDNA modified to remove the N-terminal signal peptide and to add a C-terminal His tag, and protein extracts were analysed by SDS-PAGE (upper panels) and western blots with the indicated antibodies (lower panels). Samples are from uninduced cells (lane 1), cells induced by IPTG (lane 2), induced cells after Ni affinity chromatography (lane 3), and an extract from the $S_{11}S_{12}$ containing L25 styles (lane 4). The authentic S_{12}-RNase (arrow in B) is heavily glycosylated and migrates as a doublet above the bacterially produced protein, while the authentic S_{11}-RNase runs slightly faster (arrow in A). The apparent molecular weight of protein standards (lane 5) is shown on the right. (C) The amount of radiolabel bound to anti-S_{11}- and anti-S_{12}-RNase on western blots was quantitated using a PhosphorImager for a range of both S_{11}- (closed circles) and S_{12}-RNases (open squares). S-RNase amounts shown cover the range of values measured in styles.

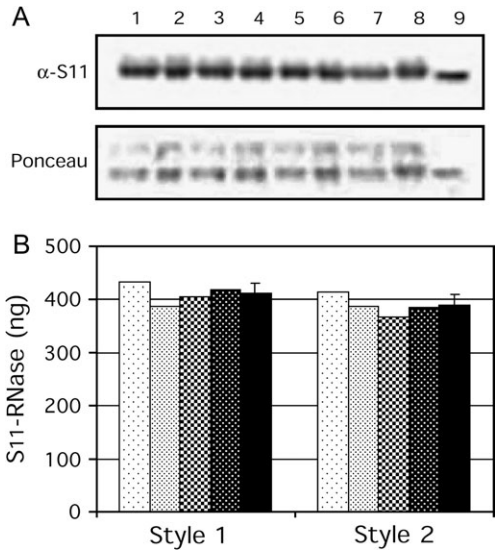

Fig. 2. Style-by-style RNase measurements are highly reproducible. (A) The S_{11}-RNase levels in quadruplicate analysis of protein extracts from two large styles (style 1, lanes 1–4; style 2, lanes 5–8) were determined by western blot analysis. An S_{11}-RNase protein standard (400 ng pure protein, lane 9) was included to standardize the measurements between gels. (B) The amount of S-RNase in each gel lane of style extract was calculated relative to the standard after quantitation of radiolabel using a PhosphorImager. For each style, the four replicate samples (stippled) and the average ±SD (black) are shown.

that a substantial difference in the average levels of S_{11}- and S_{12}-RNases can be observed in the styles of both plant lines.

It has previously been shown that average S_{12}-RNase levels in pooled stylar extracts were dependent on the plant genotype used (Qin *et al*., 2001). These observations are confirmed here using the single style measurements of S_{12}-RNase in sporadically self-compatible $S_{11}S_{12}$ lines L25 and 314 (Fig. 4A, B), the strictly incompatible S_{12} homozygote line VF60 (Fig. 4C), and the $S_{12}S_{14}$ G4 line (Fig. 4D). As expected, average S_{12}-RNase levels are low (86±55 ng) in styles from L25 plants, intermediate (136±86 ng) in those from 314 plants, and high in VF60 (269±55 ng) and G4 (301±93 ng) plant styles (Fig. 4E). More important, however, are the style-to-style variations observed within each plant genotype which here can vary up to 3-fold (Fig. 4F). Interestingly, as S-RNase levels in the fully incompatible lines VF60 and G4 can be as low as 160 ng (Fig. 4F), this level of S-RNase must lie above the minimum required for pollen rejection. The S_{12}-RNase appears as a doublet in L25 and 314 but not in G4 or VF60 due to genotype-dependent glycosylation differences (Qin *et al.*, 2001). Samples with markedly different Ponceau staining (for example, Fig. 4B, lane 5) were excluded from further analysis.

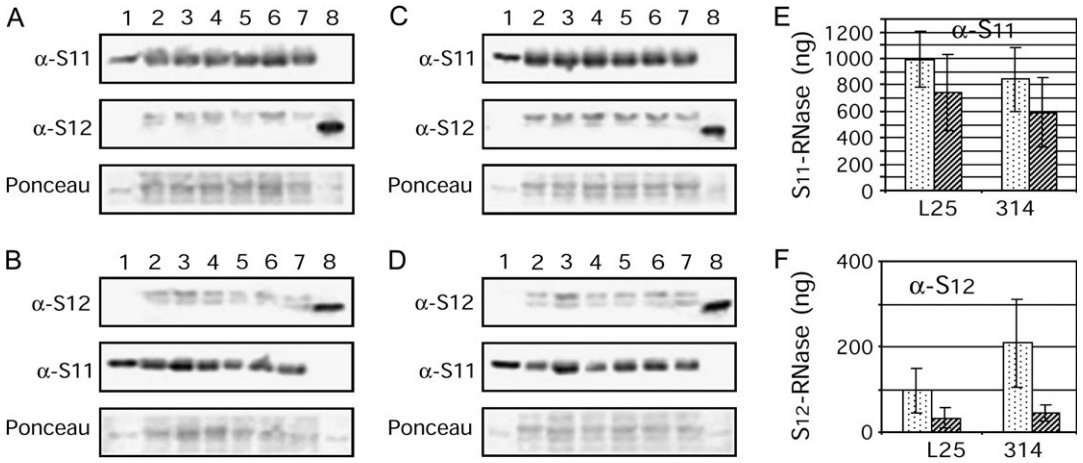

Fig. 3. Accurate S-RNase quantitation requires single-use western blots. (A) The levels of both S_{11}- and S_{12}-RNase were measured in one half of the protein extracts from single styles of $S_{11}S_{12}$ 314 plants (lanes 2–7) by sequential western blot analysis using first an anti-S_{11}-RNase then an anti-S_{12}-RNase. Standards (400 ng pure protein) for both the S_{11}-RNase (lane 1) and the S_{12}-RNase (lane 8) were included on each gel. The samples shown are a representative sample of three different gels. (B) The remaining half of the protein extracts of the 314 plants was treated as in (A) except that the order of the antibodies was reversed. (C) Analysis of one half of the protein extracts from $S_{11}S_{12}$ containing L25 plants as in (A). (D) Analysis of the remaining half the extracts from L25 plants as in (B). (E) The average ±SD ($n \geqslant 16$) S_{11}-RNase levels calculated on a per-style basis after PhosphorImager detection in styles of L25 and 314 plants when the anti- S_{11}-RNase was used first (lightly stippled bars) or second (darkly stippled bars). (F) The average ±SD ($n \geqslant 16$) S_{12}-RNase levels in styles of L25 and 314 plants determined as in (E).

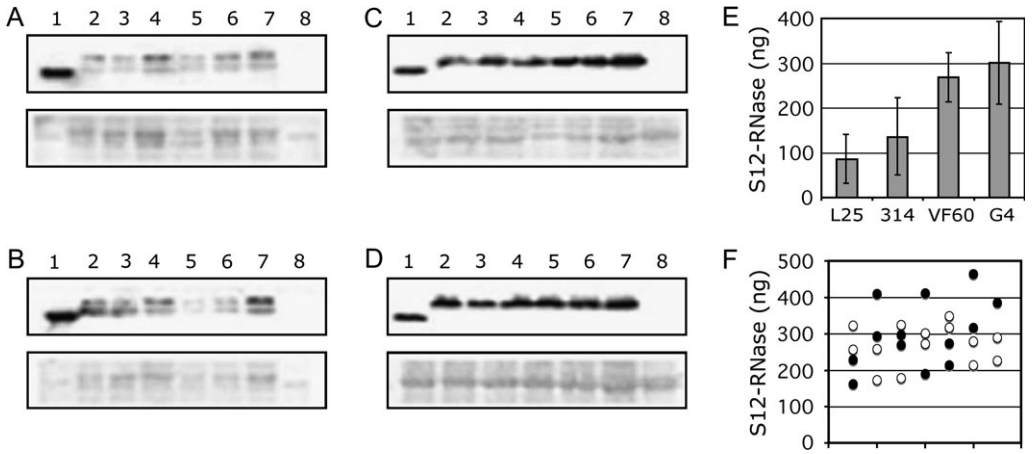

Fig. 4. S_{12}-RNase levels are genotype-dependent. (A–D) Representative western blot analysis of one half of the protein extracts from single styles (lanes 2–7) of (A) $S_{11}S_{12}$ L25 plants (n=29), (B), $S_{11}S_{12}$ 314 plants (n=44), (C) $S_{12}S_{12}$ VF60 plants (n=14), and (D) $S_{12}S_{14}$ G4 plants (n=13) were determined as described in the legend to Fig. 3. Note that S_{12}-RNase displays genotype-specific glycosylation patterns. (E) The average ±SD S_{12}-RNase levels in each of the plant genotypes. (F) The individual S_{12}-RNase levels in VF60 (open circles) and G4 (closed circles) styles. Each point represents a different S-RNase measurement, and all points were distributed horizontally so as to allow all individual points to be seen without overlap.

Experimental determination of S_{12}-RNase thresholds for pollen rejection

In contrast to the fully incompatible VF60 and G4 lines, L25 and 314 plant lines have previously been demonstrated to display a sporadic self-compatibility (SSC) phenotype (Qin *et al.*, 2001). Although the SSC phenotype has not yet been traced to a specific environmental or physiological cause, the fact that only a fraction of the L25 or 314 styles pollinated set fruit (Qin *et al.*, 2001), coupled with a large standard deviation in measured S_{12}-RNase levels, suggested that a style-by-style comparison of S_{12}-RNase levels and fruit set might allow the threshold levels required for pollen rejection to be determined.

During the course of these experiments, 14 styles were collected from L25 plants during a bout of almost complete compatibility with S_{12} pollen (31 fruits set from 33 flowers pollinated with S_{12} pollen from line 2548). The maximum amount of S_{12}-RNase in the styles sampled was 68 ng (Fig. 5A, B), and the complete compatibility phenotype during this period suggests that this amount of S-RNase must lie below the minimum level required for pollen rejection.

The S-RNase levels in individual styles described above appear to define the upper limit for pollen acceptance and the lower limit for pollen rejection, yet two problems are associated with this conclusion. First, in these cases the phenotype is complete (i.e. no pollinations produce fruits), and to determine more precisely the threshold, additional data must of necessity use incomplete phenotypes (i.e. where some pollinations are observed to set fruits). Since S-RNase levels and the incompatibility phenotype cannot

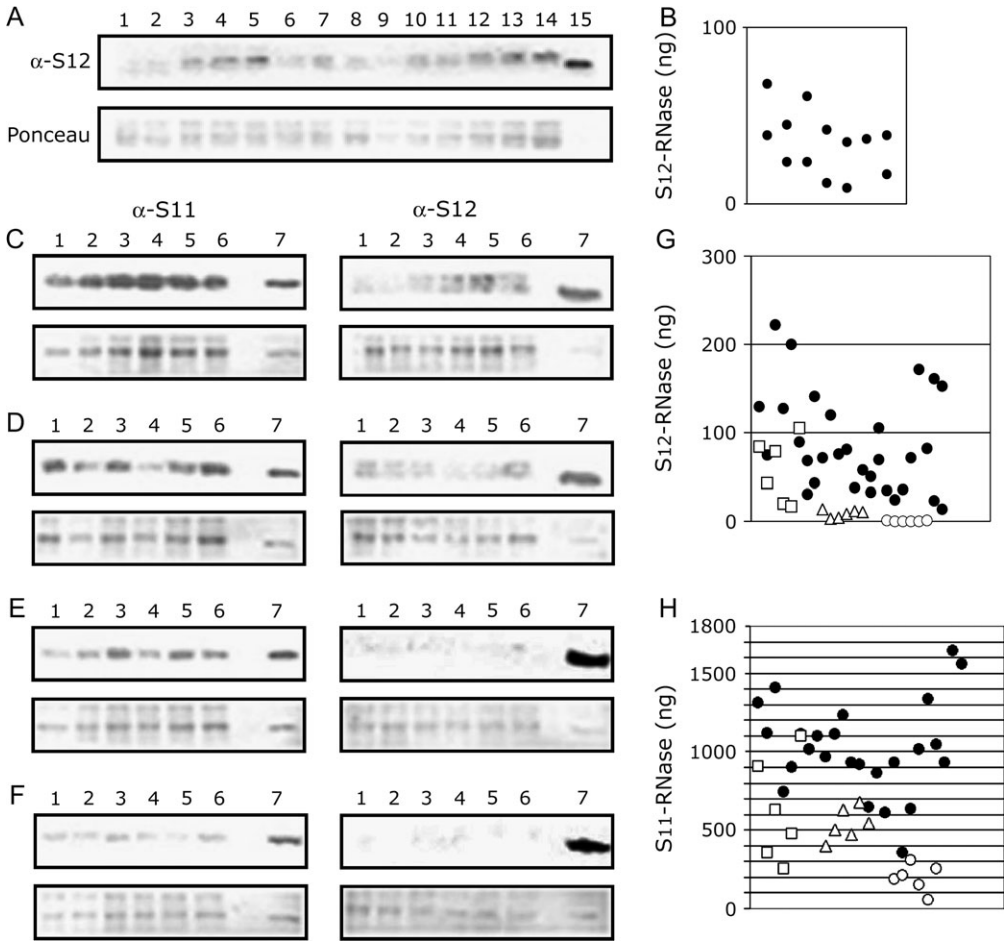

Fig. 5. S-RNase levels in L25 plants increases during stylar development. (A) Western blot analysis of one half of the protein extracts from single styles (lanes 1–14) of $S_{11}S_{12}$ L25 plants taken at a time when the plants were fully compatible with S_{12} pollen (see text). (B) The individual per-style S_{12}-RNase values of the data in (A) after quantification. The S_{12}-RNase value of lane 9 was not included because of its low protein level. Again, each point represents a single S-RNase measurement, and all points were distributed horizontally so as to allow all individual points to be seen without overlap. (C–F) Representative western blot analysis of one half of the protein extracts from single styles (lanes 1–6) of $S_{11}S_{12}$ L25 plants taken at (C) the time of flower opening, (D) 1 d before, (E) 2 d before, and (F) 3 d before flower opening using either an anti-S_{11}-RNase (left panels) or the anti-S_{12}-RNase (right panels). Protein standards in lane 7 (400 ng pure protein) are the S_{11}-RNase (left panels) or the S_{12}-RNase (right panels). Note that the contrast with the anti-S_{12}-RNase was increased using Photoshop in (E) and (F) to visualize the signals. (G) The individual S_{12}-RNase values (ng per style after PhosphorImager quantitation) are shown for single styles (distributed over the *x*-axis) taken at the time of flower opening (closed circles), 1 d before (open squares), 2 d before (open triangles), or 3 d before (open circles). Individual data points represent those contributing to the average in Fig. 4E. (H) The individual per-style S_{11}-RNase values. Data points include those from Fig. 3 where the anti-S_{11}-RNase was used first.

both be measured in the same style, correlative techniques are thus necessary. Second, it is possible that other factors might contribute to pollen rejection. It is therefore necessary to repeat these correlative experiments under several different conditions to ensure that the effect observed can indeed be ascribed to the S-RNase itself. To address these issues, S-RNase levels were examined in the SSC plant line L25 at several developmental stages. In one series of experiments, S-RNase levels were measured in individual styles of L25 plants at the time of anthesis (Fig. 5C). The average fruit set in these plants with S_{12} pollen from line 2548 is 55%, and if the S-RNase level was the principal contributor to the SI phenotype, then a threshold value of 80 ng S_{12}-RNase would leave this proportion of individual data points below the line. It was also noted that the levels of S_{11}-RNase are far above this in agreement with a full S_{11} pollen rejection phenotype if the threshold was similar (Fig. 5H).

In a second series of experiments, styles were taken between 1 d and 3 d before anthesis (DBA), a timing based on the size and morphology of the flower buds (Fig. 5D–F). Again, the predicted threshold value of 80 ng S-RNase is able to account for the incompatibility phenotype observed with S_{12} pollen from line 2548 (Table 1). It must be noted that complete compatibility with flowers pollinated at 3 DBA is not obtained, even with completely compatible pollen. This is due to the fragility of the buds, the tendency of the stylar tissues to dry after opening of the buds, and the fact that the stigma is apparently only partially receptive, as assessed by microscopic observations of fewer pollen grains that adhered and germinated.

Next, a similar series of experiments was performed with the styles taken from 314 plants. Once again, styles were analysed at anthesis (Fig. 6A) as well as between 1 d and 3 d prior to flower opening (Fig. 6B–D). The general pattern of S_{11}- and S_{12}-RNase values is similar to that observed in L25, with S_{11}-RNase high at anthesis and at 2 d prior to flower opening (Fig. 6E) while S_{12}-RNase levels were generally lower at anthesis and substantially lower 2 d prior (Fig. 6F). A predicted pollen rejection phenotype based on the number of individual styles with S_{12}-RNase levels of less than 80 ng (32%) agrees well with the observed results using S_{12} pollen from line 2548 (25%) (Table 1) suggesting that the threshold S_{12}-RNase level is similar in styles from 314 and L25 plants. Taken together, therefore, these data support the idea that a threshold of 80 ng S_{12}-RNase is sufficient to block fruit set after pollination with S_{12}-pollen.

Progeny analysis

Bud pollination of plant L25, selfed at 3 DBA, resulted in fruit formation. Plants were raised *in vitro* from the seeds and analysed by PCR using S-allele-specific oligos to assess whether the three possible genotypes ($S_{11}S_{11}$, $S_{11}S_{12}$, and $S_{12}S_{12}$) were present in the expected ratios. Of the 202 F_1 progeny, 90 were $S_{11}S_{12}$, 112 were $S_{12}S_{12}$, and none were $S_{11}S_{11}$ (x^2=59.17, P <0.001). It is concluded that S_{11} pollen is fully rejected even at 3 DBA, despite the observation of low (53 ng) S_{11}-RNase levels measured in one out of six styles analysed (Fig. 5). It is possible that the threshold for S_{11}-RNase is lower that the 80 ng threshold estimated for S_{12}-RNase. Alternatively, the steady increases in S_{11}-RNase observed between 3 and 2 DBA may be sufficient to block S_{11} pollen tubes before they reach the ovary.

S_{12}-RNase thresholds differ for different pollen types

The S_{12}-RNase threshold estimated from pollen rejection phenotypes using pollen from plant 2548 is similar when styles of L25 and 314 plants are compared (Table 1). However, this experiment does not address the potential influence of the pollen itself on the estimated thresholds. Thus, in another experiment, the pollination efficiency of four types of pollen from different genetic background was compared on L25 styles. All these pollen show similar viability (based on their appearance after staining with acetocarmine) and good germination. Despite this, major differences are observed in pollination efficiency (Table 2), and this suggests that the S-RNase thresholds may be different for the different types of pollen. Indeed, if thresholds are estimated as before by placing an arbitrary threshold line in Fig. 5G at a value where the proportion of

Table 1. *Comparison of S_{12}-RNase levels and S_{12} pollen rejection phenotype*

	Plant line L25			Plant line 314		
	Average ng S_{12}-RNase[a]	No. styles ⩽80 ng S_{12}	No. fruits/ no. flowers	Average ng S_{12}-RNase[b]	No. styles ⩽80 ng S_{12}	No. fruits/ no. flowers
Anthesis	86±55	17/30 (57%)	44/80 (55%)	136±96	14/44 (32%)	25/98 (25%)
1 DBA	58±37	4/6 (66%)	7/14 (50%)	66±10	5/6 (83%)	19/24 (79%)
2 DBA	9±4	6/6 (100%)	16/17 (94%)	26±16	6/6 (100%)	11/12 (92%)
3 DBA	0.4±0.3	6/6 (100%)	8/11 (73%)	16±7	6/6 (100%)	7/10 (70%)
Anthesis[c]	37±18	13/13 (100%)	31/33 (94%)			

[a] Data are the average ±SD of the points in Fig. 5G.
[b] Data are the average ±SD of the points in Fig. 6E.
[c] Data are the average ±SD of the points in Fig. 5B.

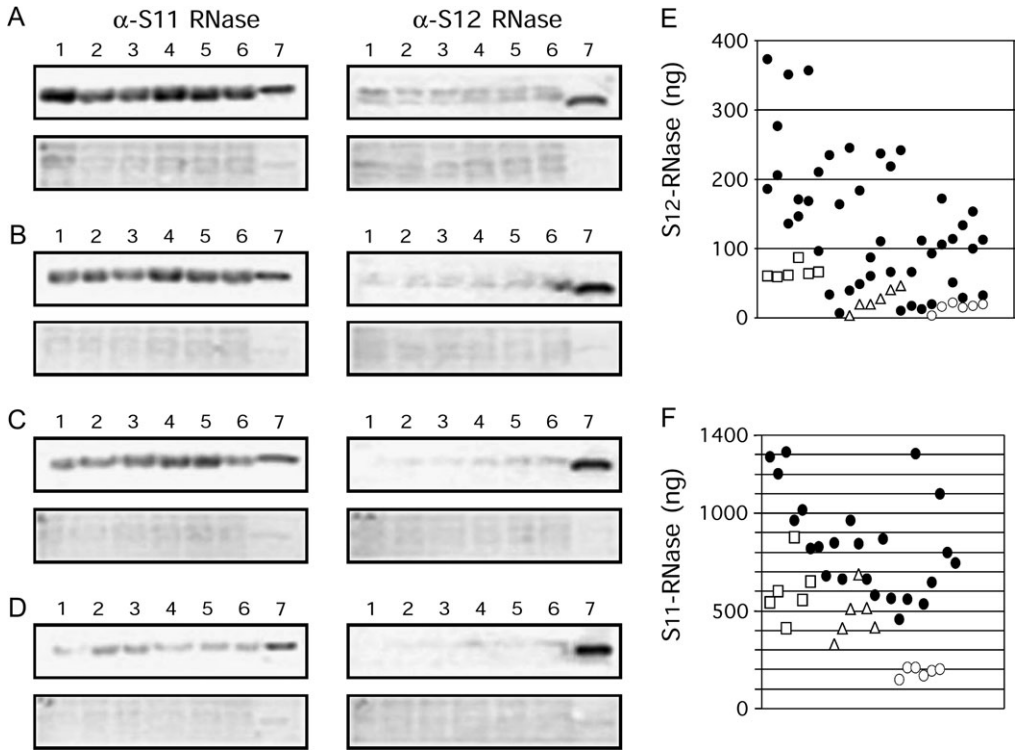

Fig. 6. S-RNase levels in 314 plants increases during stylar development. (A–D) Western blot analysis of one half of the protein extracts from single styles (lanes 1–6) of $S_{11}S_{12}$ containing 314 plants analysed as in the legend to Fig. 5. (E) The individual per style S_{11}-RNase values (ng per style after PhosphorImager quantitation) are shown for plants taken at the time of flower opening (closed circles), 1 d before (open squares), 2 d before (open triangles), or 3 d before (open circles). Data points include the first anti-S_{11}-RNase in Fig. 3. (F) The individual per style S_{12}-RNase values presented as in (E). Individual data points represent those contributing to the average in Fig. 4.

Table 2. *Threshold S_{12}-RNase levels depend on pollen genetic background*

Staminate parent	Pollen haplotype	No. fruits/ no. flowers[a]	Est. S_{12} RNase threshold[b]	Pollen stainability
TP48	S_{12}	32/39 (82%)	130 ng	>90%
2548[c]	S_{12}	44/80 (55%)	80 ng	>90%
VF60	S_{12}	10/50 (20%)	35 ng	>90%
L25	S_{11} and S_{12}	5/68 (7%)	25 ng	>90%

[a] All crosses used L25 line as pistillate parent.
[b] Determined from the S_{12}-RNase levels in Fig. 5G.
[c] Data as in Table 1.

styles with lower S-RNase levels corresponds to the percentage fruit set, the estimated S_{12}-RNase threshold differs by up to 5-fold (Table 2). It is concluded from this that the S-RNase threshold must be defined for each particular pollen type.

Microscopic observations

To complement the fruit set measurements on genotypes L25 and 314, microscopic observations were also made to assess the behaviour of pollen tubes inside the styles 2–3 d after pollination. Following self-pollination, S_{12} pollen tubes were typically observed in the middle or lower third of the style, unlike S_{11} pollen tubes, which all arrested in the upper third of the style. Following pollination with S_{12} pollen from VF60 or plant 2548, however, a large variation was found in the number of pollen tubes observed at the stylar basis. In some cases, numerous pollen tubes were observed to have entered the ovary, and the appearance of the stylar bases were indistinguishable from compatible pollinations (usually yielding about 120 seeds) (Fig. 7A). This was interpreted as being consistent with a low level of S_{12}-RNase in these styles. In other cases, no tubes were observed at the stylar base, which was interpreted as consistent with a high level of S-RNase (Fig. 7B). In other instances, a reduced number of pollen tubes (from one up to ten, but most often one or two) can be seen to have reached the base of the style and to have entered the ovarian region. Some of these pollen tubes may arrest just after their entrance into the ovarian region, while others can be observed to penetrate inside the ovules (Fig. 7C, D). Curiously, however, fruits containing only one or two seeds were never observed. Indeed, the smallest numbers that were ever observed were four and five seeds in two different fruits and, in both cases, they were accompanied by numerous aborted seeds and swollen ovules.

These observations suggested that a single pollen tube reaching the ovary may be insufficient to allow fruit set, and

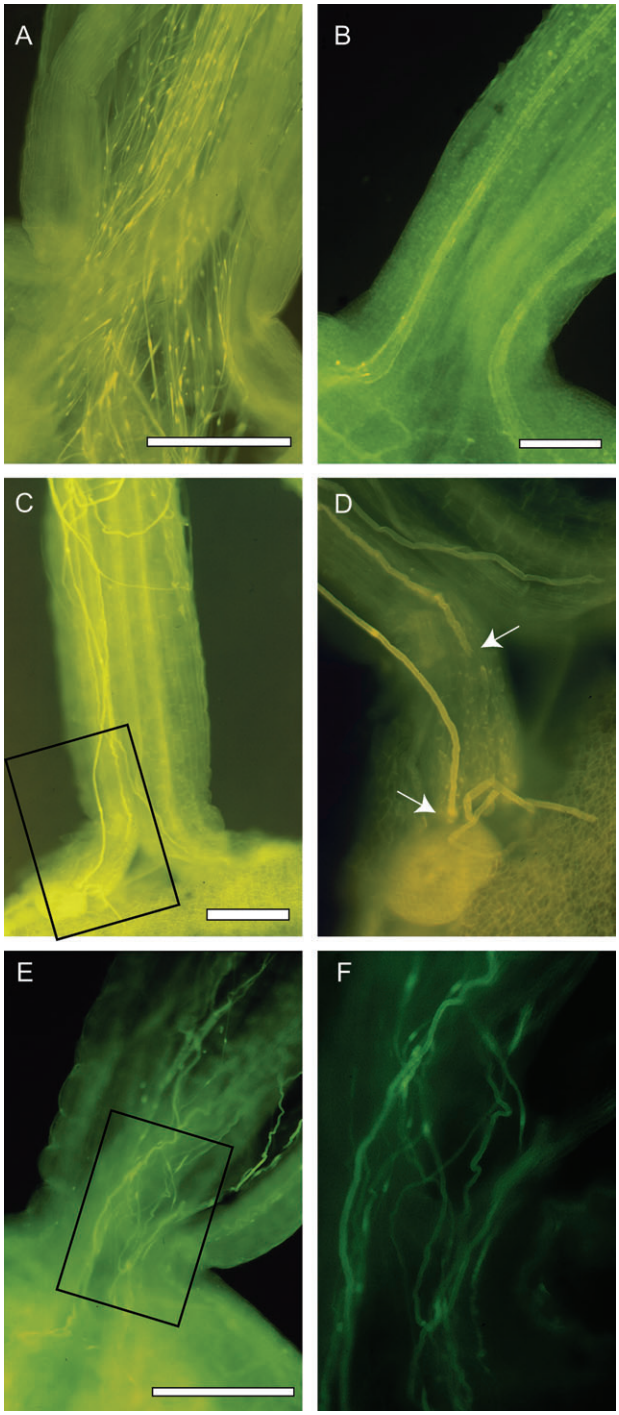

Fig. 7. Fruit set requires a threshold number of pollen tubes entering the ovary to accomplish fertilization. (A–F) Pollen tubes in the base of L25 styles visualized by aniline blue staining after pollination with S_{12} pollen from plant 2548. (A) A pollination resembling a compatible cross. (B) A pollination resembling a fully incompatible cross. (C, D) Pollination with a few pollen tubes reaching the ovary. The inset in (C) has been magnified in (D) to show both an arrested pollen tube at the ovarian entrance (upper arrow) and a pollen tube entering an ovule (lower arrow). Also note the pollen tubes arrested earlier at the top of the picture, which represents the lower third of the style. (E, F) Stylar observation of an incompatible pollination 5 d post-pollination. The inset in (E) has been magnified in (F) to show 10 pollen tubes clearly entering the ovarian region. Bars represent 0.5 mm.

that assessment of SI either by number of pollen tubes at the stylar base or by fruit set may differ. To test this, the simple expedient of collecting, 4–5 d after pollination of L25 or 314 plants with S_{12} pollen, flowers that would fall when gently shaken was used. These incompatible crosses were then examined microscopically to determine the number of pollen tubes at the stylar base. Although in many cases the pollen tubes were all arrested in the middle or lower third of the style, instances were also found where up to ten tubes could be found entering the ovarian region (Fig. 7E, F). It was deduced from this that fruit formation may require more than 10 pollen tubes entering the ovarian region, although it was noted as a caveat that this value may include some slow growing tubes that reach the ovary after the process leading to flower abscission has already been initiated. As a complement to these studies, 24 styles of L25 plants pollinated during its bout of almost complete self-compatibility were also examined at 72 h post-pollination. While 21 styles had 20 or more (in most cases, uncountable) pollen tubes in the ovarian region, in the remaining cases only 14 (two cases) or 15 (one case) pollen tubes were found in the ovarian region. Since the ovarian regions contained 14 pollen tubes in these compatible crosses, and ten in the incompatible crosses described above, these results suggest that a number of pollen tubes in excess of this 10–14 tubes threshold must enter the ovary to ensure fruit formation.

Discussion

Style-to-style variations in S-RNase levels can be used to estimate the S-RNase threshold

A considerable natural variability in the amount of S-RNases present in individual styles has been found. The extent of this variation was surprising, as individual styles can differ in S_{12}-RNase levels by over 20-fold. This phenomenon has not previously been reported, and it was observed here only because S-RNase levels had been measured for individual styles. Interestingly, these variations neatly account for the observations reported here in which fruit set for an individual style is an all-or-none phenomenon but where different styles on the same plant may behave differently. It is not believed that these variations represent technical problems with the assay since our standard curves are linear (Fig. 1) and replicates from the same biological samples have a low coefficient of variation (Fig. 2).

These variations in individuals can be exploited to estimate the threshold below which S-RNases are ineffective in pollen rejection. For example, when an S-RNase expressing plant is completely compatible (Fig. 5B), the highest stylar level found (68 ng) defines the highest point still below the threshold. Similarly, when the plant is completely incompatible (Fig. 4F), the lowest value of S-RNase

obtained (160 ng) defines the lowest point still fully capable of pollen rejection.

Do these values truly reflect the existence of an S-RNase threshold? This question is not trivial, as other factors such as HT-B (O'Brien *et al.*, 2002) or a stylar 120 kDa glycoprotein in *Nicotiana* (Hancock *et al.*, 2005) have been shown to be required for pollen rejection. However, if a factor other than the S-RNase were to contribute substantially to pollen rejection, a repeated correlation between incompatibility and S-RNase levels would be unlikely. It is thus significant that the thresholds estimated from two different plants lines (L25 and 314) under all these conditions are so similar. Furthermore, the same threshold accounts for the acquisition of the incompatibility phenotype in developing flowers (Figs 5G, 6E), long cited as support for the threshold hypothesis itself. Taken together, it is proposed that the numerous times the same correlation is observed provides strong support for the idea that S-RNases play the primary role in determining the SI phenotype. If so, this in turn suggests that the S_{12}-RNase threshold required for pollen rejection is indeed around our value of 80 ng per style.

S-RNase concentrations inhibiting pollen tube growth are remarkably similar in different plants

The value proposed here for the threshold of the S_{12}-RNase in *S. chacoense* should be considered in the context of the average amounts of S-RNase present in the styles of other fully self-incompatible Solanaceae genotypes. Average S-RNase levels have appeared in a number of different reports, and it must first be noted that these values can differ markedly depending on the method used. For example, calculations of the RNase levels in S_2S_2 homozygous self-incompatible *N. alata* based on the amount of S-RNases purified from styles yielded estimates of 10 μg (Jahnen *et al.*, 1989*a*, *b*) or ~20 μg (Gray *et al.*, 1991) S_2-RNase per style. By contrast, measurements based on comparisons of staining intensity of an S_2-RNase band after electrophoresis of crude extracts with that of the purified S-protein, yielded estimates of 90 μg S_2-RNase per style in the same plants (Harris *et al.*, 1989). The lower levels calculated after protein purification may perhaps be attributable to losses during purification. Alternatively, the higher levels found after electrophoresis may have been due to the presence of contaminating proteins with the same electrophoretic mobility as the S_2-RNase. This study's protocol, which compares immunoreactivity in crude extracts with those of a pure standard S-RNase, appears less susceptible to experimental errors of either type. Our values about 1 μg S_{11}-RNase and 0.1 μg S_{12}-RNase in the styles of L25 plants (Fig. 3) appear lower than that found in *Nicotiana*, but it must be borne in mind that *Nicotiana* styles (~15 mg) weigh roughly ten times more than *S. chacoense* styles (~1.3 mg). Indeed, the calculated concentrations of S-RNases in styles of *S. chacoense* (0.25–1 mg ml^{-1}) and *N. alata* (0.5–5 mg ml^{-1}) are quite similar.

The calculation of S-RNase thresholds *in vivo* is difficult, as in general the genotypes studied are fully incompatible and the degree to which their S-RNase levels surpass the threshold can therefore not be ascertained. Even in plants where some degree of compatibility is observed, measurements of S-RNase levels and incompatibility phenotype in an individual style are mutually exclusive. Methods employing correlations in populations of styles are thus required. One such approach has been taken with the Japanese pear (Rosaceae), where some cultivars can be up to 15% compatible (Hiratsuka and Zhang, 2002). Since the S-RNase values vary 2-fold between different plant genotypes, and the fully incompatible S_3-RNase is present at roughly 0.2 mg ml^{-1} in styles of the fully incompatible cultivar 'Choiuro' (Matsuura *et al.*, 2001), this suggests that the threshold may lie close to 0.1 mg ml^{-1} (Hiratsuka and Zhang, 2002). This value is remarkably similar to the 80 ng (0.06 mg ml^{-1}) per style estimated from the studies reported here.

Genotypic differences in expression of style and pollen components of SI

The observation that different S-RNases are expressed to different levels in styles of a given plant (Fig. 3) leaves open the important question of the expression of the same S-RNase in different plant genotypes. In general, an effect of the genotype of the donor plant on the amounts of the S-RNase it produces, i.e. the level of expression of the same S-RNase in different genetic backgrounds, has only rarely been considered. In one study, the expression of S_{A2}-RNase constructs in different *Nicotiana* species was found to depend on the genetic background of the host (Murfett and McClure, 1998). In another study using Japanese pear, the same *S*-allele produced different amounts of S-RNase depending upon the cultivars (Zhang and Hiratsuka, 1999). In *S. chacoense*, a comparison of the levels of S_{11}-RNase in genotypes L25 and 314 suggests that only slight differences may be observed (Fig. 3E). However, in the case of the S_{12}-RNase, up to 3-fold differences were observed among the four genotypes tested (Fig. 4). For the S_{12}-homozygote line VF60, at least part of the increase may be related to the number of S_{12} genes present. However, this explanation cannot account for the high levels in the $S_{12}S_{14}$ heterozygote G4 line. In this regard it is interesting to note, in various cultivars of Japanese pear, the systematic (almost exclusive) association of S-RNases (S_1, S_3, S_5) that are both more abundant in the styles and yield strong SI phenotypes, with those (S_2, S_4, S_6 and S_7) with weaker SI phenotypes and whose abundance in the styles is reduced (Zhang and Hiratsuka, 1999; Hiratsuka and Zhang, 2002). It was concluded that the strength of the SI system in the various cultivars depends on the total

S-RNase content rather than the levels of individual S-RNases (Zhang and Hiratsuka, 1999).

The genetic background of the pollen may influence the S-RNase threshold

In addition to genotype-dependent differences in expression of the stylar component to SI, these analyses also suggest that differences may be found in expression of the pollen component. For example, when pollen of different staminate genotypes was tested on the same pistillate parent, the proportion of fruits set and the calculated S_{12}-RNase thresholds were considerably different (Table 2). Around a 4-fold difference was calculated in the S_{12}-RNase thresholds for pollen from the three S_{12}- homozygous individuals tested, similar to the over 3-fold differences observed for S_{12}-RNase expression in different genotypes (Fig. 4). Although the difference in the calculated threshold of L25 pollen appears even lower, it must be noted that only half the pollen derived from the L25 pollen has the S_{12} haplotype. It is tempting to speculate that expression levels of the pollen component to SI may also vary according to the genetic background, and that these variations may be responsible for the differential sensitivity of the pollen to its cognate S-RNase. Alternatively, it is possible that the different pollen types tested here differ in their S_{12}-RNase uptake efficiency. These factors can be scrutinized when the pollen component is finally identified in *S. chacoense*.

Consequences of an S-RNase threshold for conceptualizing SI

The determination of what appears to be a relatively sharp S-RNase threshold has some important implications that have not previously been made explicit in studies on self-incompatibility. The reason for this is that several molecular mechanisms have previously been reported to give rise to threshold phenomena, also termed ultrasensitive or switch-like. For example, ultrasensitive responses can be due to co-operative interactions between subunits, to a requirement for multiple phosphorylation events, or to covalent modifications carried out by modifying enzymes working at saturating substrate levels (zero-order kinetics) (Ferrell, 1998). The mechanism leading to the threshold phenomenon in GSI is presently unknown, but to date the only covalent modification suspected is ubiquitination, and as S-RNase levels must rise in the pollen after import from the style they will not be initially saturating. However, as it has previously been proposed that pollen S may function as a multimer (Luu *et al.*, 2001), one attractive possibility is that co-operative interactions might also exist between S-RNase subunits. Indeed, it would be of interest to examine the kinetics of purified S-RNases *in vitro* for evidence of co-operative behaviour. A second intriguing possibility is that the threshold might reflect the presence of stoichiometric levels of a factor that binds with high affinity to the RNase. If true, this suggests that biochemical approaches might be successful in isolating the factor responsible.

Fruit formation requires a minimum number of fertilization events

These results suggest that more than 10 pollen tubes entering the ovary and accomplishing fertilization may be necessary to trigger fruit formation and sustain its subsequent development. This facet of the SI phenotype has been revealed by observations by fluorescence microscopy of styles from flowers that drop, after gentle shaking, 4–5 d after pollination. Since in SI studies a cross is generally considered incompatible when pollinated flowers drop, the presence of up to 10 pollen tubes entering the ovary seems surprising. It is known that a minimum number of fertilization events is required for fruit set and its subsequent growth (Gillaspy *et al.*, 1993; Hiratsuka and Zhang, 2002), and this is consistent with the finding that when fruits contained only a few well-developed seeds, numerous aborted seeds or swollen ovules were always present. This is similar to what was observed in other species, for example in *Lycopersicon* (de Nettancourt *et al.*, 1974; Gradziel and Robinson, 1989). However, it is difficult to assess if this may be more generally true, as reports of fruits with only a single seed (de Nettancourt and Ecochard, 1968; de Nettancourt *et al.*, 1971) made no mention of either the presence or absence of aborted seeds, nor were microscopic observations performed in those studies. In addition, it is important to recognize that the entrance of pollen tubes into the ovarian region represents only an estimation of the outcome of the cross, as the number of pollen tubes actually accomplishing fertilization is the determinant factor for fruit set. It is known that environmental factors such as temperature, heat, humidity etc. can affect self-incompatibility, although it seems unlikely that these factors would selectively affect only some styles on a plant as was observed here.

The number of fertilization events required for fruit formation has a particularly interesting implication for the evolution of the SI system. It has long been recognized that mutations of *S*-alleles occur in nature, yet attempts to produce new *S*-alleles by mutagenic treatments have not been successful (de Nettancourt, 1977, 2001), and have only been observed after site-directed mutagenesis (Matton *et al.*, 1997, 1999). These results suggest that, even if pollen mutants were generated experimentally, these mutations could not be transmitted to the progeny upon experimental selfing if a single mutant pollen grain is insufficient to allow fruit formation, unless hormone treatments were applied to the pollinated flowers in order to prevent premature abscission (de Nettancourt *et al.*, 1971; Golz *et al.*, 1999). By contrast, under open pollination conditions such as occur in nature, the concomitant pollination with a mutant pollen and abundant compatible pollen from neighbour

plants would allow fruit set to occur normally, in turn allowing the mutant zygote to develop and begin to spread the mutant genotype within the population.

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