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Self-compatibility in a *Lycopersicon peruvianum* variant (LA2157) is associated with a lack of style S-RNase activity

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Abstract A series of crosses between a naturally-occurring self-compatible accession of *Lycopersicon peruvianum* and a closely-related self-incompatible accession were used to demonstrate that the mutation to self-compatibility is located at the S-locus. Progeny of the crosses contain abundant style proteins of about 30 kDa that segregate with the S_6- and S_7-alleles from the SI parent and the S_c-allele from the SC parent. The S_6- and S_7-associated proteins have ribonuclease activity whereas the S_c-associated protein is not an active ribonuclease. This finding indicates that S-RNases are determinants of self-incompatibility in the style and that the ribonuclease activity is essential for their function.

Key words Tomato · *Lycopersicon peruvianum* Self-incompatibility · S-locus mutation · Pollination Style-ribonuclease (S-RNase)

Introduction

Gametophytic self-incompatibility (GSI) is a genetically-based mechanism that operates to prevent self-pollination, and thus inbreeding, in many angiosperm taxa (Mau et al. 1991). Self-incompatibility (SI) in the Solanaceae is controlled by a single locus, the S-locus, that has multiple alleles. When the allele expressed by the haploid pollen grain is also expressed in the female tissue of the style, pollen-tube growth is inhibited within the style thus preventing fertilization of the ovules. The onset of self-incompatibility in the style of solanaceous plants is associated with the synthesis of glycoproteins with ribonuclease (RNase) activity. These proteins are found in the extracellular spaces of the transmitting tract and, because different forms of these proteins co-segregate with alleles of the S-locus, they are referred to as S-RNases (for reviews see Clarke and Newbigin 1993; Newbigin et al. 1993; Sims 1993). Comparison of the sequences of 20 different S-RNase alleles shows a characteristic structure which includes five tightly-conserved regions, two of which are probably associated with the active site of the RNase, and other regions that are highly variable between alleles and may be involved with allelic specificity (Ioerger et al. 1990; Tsai et al. 1992). The RNase can enter pollen tubes growing in vitro (Gray et al. 1991) and, although no allele specificity was detected in the in vitro assay, these RNases may be responsible for the degradation of RNA observed in incompatible pollen tubes, but not in compatible pollen tubes growing in vivo (McClure et al. 1990). The nature of the S-gene product in pollen is not established. Although the S-RNase gene is also expressed in developing pollen (Dodds et al. 1993), there is no evidence for the involvement of these molecules in determining the SI phenotype of pollen.

Sequence analysis of S-RNases, and the use of these sequences to detect related genes in other species, indicate that at least some of the S-alleles arose before the speciation of the Solanaceae (Ioerger et al. 1990; Rivers et al. 1993). This is consistent with the idea that GSI is a primitive trait and that self-compatible plants arose from self-incompatible plants by alteration of an essential component required for the recognition or rejection of self-pollen (Whitehouse 1951; Jain 1976; Mayo and Leach 1987; Rick 1982 a). The present paper describes a self-compatible variant that was present near a population of self-incompatible *Lycopersicon peruvianum* plants in Peru. The mutation segregates with the S-locus and is associated with low style-ribonuclease activity, suggesting that the ribonuclease is an essential element of self-incompatibility in solanaceous plants.

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Materials and methods

Plant material

L. peruvianum (L.) Mill. accessions LA 2157 (S_cS_c, self-compatible) and LA 2163 (S_6S_7, self-incompatible) were collected in the Rio Chotano valley, Cajamarca, in northern Peru by Dr. C. Rick (Tomato Genetics Resource Center, University of California, Davis). LA 2157 was growing near Choto and LA 2163 was found further north near Huambos (Rick 1982 b). Plants were grown from seed in an insect-free glasshouse, and an individual of each accession was maintained by vegetative propagation for use in subsequent breeding experiments. Tester plants, homozygous for the S_7- or the S_6-allele, were obtained by self-pollination of a heterozygous individual (S_6S_7) at the green-bud stage, using a light coating of 1% α-napthaleneacetic acid (NAA) in crude lanolin smeared on the floral pedicels to prevent abscision of poorly-filled fruits. Progeny grown from the bud self-pollination were identified as homozygous for the S_7- or S_6-allele based on incompatibility with pollen from the parent S_6S_7 plant and cross compatibility with each other. The homozygous plants were used as pollen parents in subsequent breeding experiments to assign the S-genotype.

Breeding experiments

Inheritance of the self-compatibility trait was monitored in progeny arising from three mating experiments. The F_1 population was produced by crossing individuals from the LA2163 and LA2157 accessions, using LA2163 (SI) as the pistillate parent. An F_1 individual with an S_7S_C genotype (denoted $F_1S_7S_C$) was self-pollinated to produce the F_2 population and backcross progeny of $F_1S_7S_C$ were produced by reciprocal pollination with LA2163. All crosses were performed in the glasshouse using standard emasculation and pollination techniques. The emasculation procedure was altered for the determination of the compatibility status of the progeny of the crosses because the entangled epidermal hairs on the style and anthers led to difficulties in removal of the anthers without damage to the pistil. One-day prior to petal opening, petals were removed and anthers were cut halfway along their length with scissors. Lanolin paste was smeared on the anthers and around the style, but not the stigma, and the flower was bagged. The following morning the pistil was hand-pollinated with self-pollen or pollen from the S_7S_7 or S_6S_6 tester stock and the flower was rebagged. Seven-to-ten-days later, the flowers were debagged and examined. Flowers that were attached to the pedicle were designated compatible and detached flowers were scored as incompatible. Three to five pistils were examined for each pollination.

Pollen-tube growth

Pollen-tube growth in the pistils of the parent plants (LA2163 and LA2157) was monitored 48 h after pollination as described by Maheswaran et al. (1986). Pollinations were judged incompatible when pollen tubes were arrested in the upper third of the style and compatible when the pollen tubes reached the ovary.

Protein-gel electrophoresis and RNase-activity gels

Pistil extracts were prepared by grinding ten styles with a plastic tissue homogenizer in 1.5-ml microcentrifuge tubes containing 20 µl of extraction buffer [0.1 M Tris/HCl, pH 7.8, 10 mM EDTA and 2% (w/v) Polyclar AT] at 4 °C. The supernatant was collected after centrifugation at 12 000 rpm for 15 min at 4 °C and protein concentration was determined using the Coomassie blue protein assay (BioRad) with bovine serum albumin as a standard. To minimise pollen contamination of the pistils, flowers were collected 1-day prior to anthesis and placed upright in 0.8% (w/v) agar containing 3% (w/v) sucrose. Pistils were collected the following day when the flowers had opened.

For SDS-polyacrylamide-gel electrophoresis, samples containing 1.5 µg of protein were mixed with an equal volume of a solution containing 50 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and 0.05% (w/v) bromophenol blue and were heated at 100 °C for 5 min and cleared by centrifugation prior to electrophoresis on 15% (w/v) acrylamide gels as described by Laemmli (1970). Proteins were detected by staining with silver reagent (BioRad), and protein sizes were estimated by comparison to known standards (Pharmacia).

For RNase activity-gels, style proteins (30 µg in 12 µl) were mixed with 3 µl of 100 mM Tris/HCl, pH 6.8, 5% (w/v) SDS, 25% (v/v) glycerol and 0.06% (w/v) bromophenol blue and were not heat treated before they were loaded onto the gel. Electrophoresis and activity staining was performed on SDS-acrylamide gels as described by Yen and Green (1991). The separating gel contained 15% (w/v) acrylamide and RNase activity was detected following incubation at 37 °C in 0.1 M Tris/HCl, pH 7.0, containing 10 mM EDTA for 1 h.

Results

Morphological characteristics of self-compatible (LA2157) and self-incompatible (LA2163) accessions of *L. peruvianum*

Both LA2163 and LA2157 have a simplified leaf structure composed of three pairs of major leaflets, one terminal leaflet and 6–8 minor leaflets. The leaflets are serrate and the flower trusses are unbranched (Fig. 1 A, B). The two accessions are characterized by thin and wiry stems and have small flowers relative to accessions from other areas. There is no marked difference in flower size between the two accessions.

Determination of self-incompatibility status

The self-incompatibility status of the two accessions was established by monitoring pollen tube growth and fruit set after self-pollination. Pollen tubes in self-pollinated LA2163 were arrested one-third to one-half of the way down the style and often had the abnormal callose deposits and swollen tips that are characteristic of incompatible pollen tubes. In contrast, self-pollen of LA2157 grew directly to the ovary where successful fertilization and fruit set occurred (Fig. 1 C). Seed set in the fruit of the self-pollinated LA2157 accession was as successful as seed set following cross-pollination in LA2163; that is, each fruit contained 20–30 viable seeds.

Inheritance of self-compatibility in breeding experiments involving LA2157 (SC) and LA2163 (SI)

Several cross pollinations were performed in the genetic analysis of self-compatibility in the LA2157 accession (see Table 1). An F_1 population of 20 plants was produced from the LA2163 (S_7S_6)×LA2157 (S_cS_c) cross. All F_1 plants contained an S_c-allele since they were all self-compatible and compatible with both parents. They were divided into two groups according to their ability to reject pollen from the homozygous plants, S_7S_7 or S_6S_6. Seven plants, designated S_7S_c, rejected S_7-pollen and 13 plants (S_6S_c) rejected the S_6-pollen. The segregation ratio was 1:1 ($\chi^2 = 1.8$, $P > 0.1$).

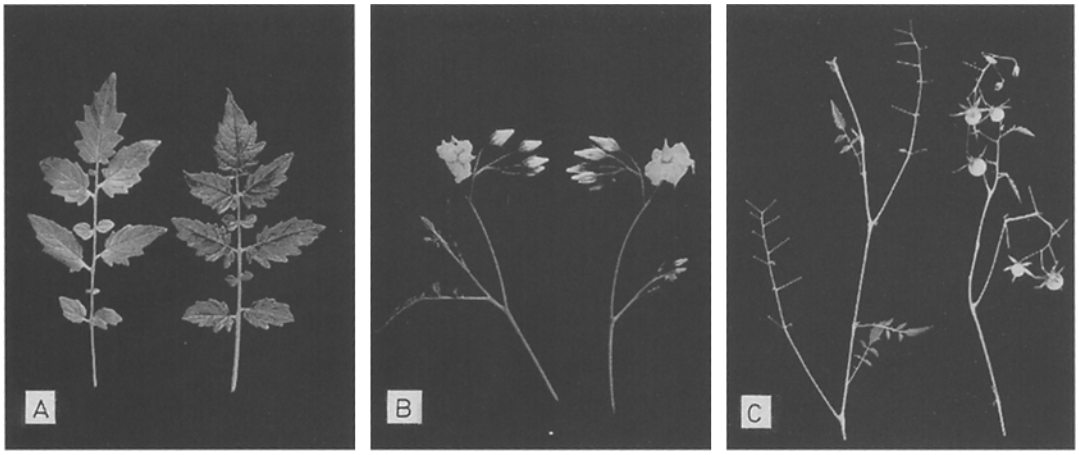

Fig. 1A, B, C Leaf, flower cluster and fruit of LA 2163 (S_6S_7) and LA 2157 (S_cS_c). **A, B** The leaves and flower clusters of the self-incompatible plant LA2163 (left) and the self-compatible mutant LA2157 (right) have similar morphology. **C** Only the self-compatible mutant (right) sets fruit after self-pollination

An F_2 population was produced by self-pollination of an individual with the S_7S_c genotype ($F_1S_7S_c$) and 37 progeny were examined. All were self-compatible and fell into two groups. One group, containing 20 individuals, was fully compatible with pollen from the S_7S_7 and S_6S_6 testers and was assigned the S_cS_c genotype. All individuals (17) in the other group rejected S_7-pollen, accepted S_6-pollen and were assigned an S_7S_c genotype. The segregation ratio was 1:1 ($\chi^2 = 0.24$, $P > 0.5$).

A backcross generation (BC_1-1) was obtained from the $F_1S_7S_c \times$LA 2163 (S_7S_6) cross. Of the 28 progeny 10 were SI and 18 were SC. The SI plants were assigned the S_6S_7 genotype on the basis of their ability to reject pollen from both the S_7S_7 and S_6S_6 tester plants. All the SC plants rejected S_6-pollen and accepted S_7-pollen and were consequently designated as S_6S_C genotype. Segregation of the two groups was in a 1:1 ratio ($\chi^2=2.28$, $P > 0.1$). In the reciprocal cross, LA 2163 (S_7S_6)$\times F_1S_7S_c$, 40 progeny (BC_1-2) were obtained. All were self-compatible.

Identification of S_7-, S_6- and S_c-associated proteins in the styles of *L. peruvianum*

Style extracts from the parent plants, plants homozygous for the S_7-, S_6- or S_c- alleles, and plants from the F_1, F_2 and BC_1 populations, were examined by electrophoresis on SDS-polyacrylamide gels followed by silver staining. Extracts from all genotypes contained abundant proteins with a molecular mass of about 30 kDa that segregated with the S-genotype (see Fig. 2 for representative data). Plants assigned the S_6-genotype on the basis of their breeding behaviour, contained a 30.4 kDa protein whereas proteins of 29.7 kDa and 28.6 kDa, respectively, were associated with the S_c- and S_7-alleles.

RNase-activity gels of style extracts from homozygotes and an F_2 population segregating for the S_7-allele

Figure 3 shows an RNase-activity gel of style extracts from S_6S_6, S_7S_7 and S_cS_c homozygous plants and the S_6S_7 heterozygote.The extracts contained four to six ribonucleases that migrated as distinct bands on the RNase-activity gels. Four of the bands (15.8, 18.6, 24.5 and 56.2 kDa) were common to all styles irrespective of S-genotype . All plants with the S_7-genotype had an additional major band of about 28 kDa, while all plants with the S_6-genotype had a band of 27 kDa. These RNases correspond to the allele-specific proteins observed in Fig. 2. The slight differences in mobility were due to differences in sample preparation and gel composition. In the S_6S_7 heterozygote, two bands corresponding to the products from the S_7- and S_6-alleles were detected, with the S_7-band predominating. Activity gels of style extracts from plants in the F_2 population that were segregating for the S_7-allele showed a major band of RNase activity of about 28 kDa that was present at a much reduced level in extracts of S_cS_c plants (Fig. 4). None of the

Table 1 Crosses used for genetic analysis of the self-compatible (LA2157) and self-incompatible (LA2163) plants

Cross pollination				Progeny after cross	Expected genotypes and segregation ratio	Observed segregation ratio	χ^2	P
Female		×	Male					
P_1	(LA2163, S_6S_7)	×	P_2 (LA2157, S_cS_c)	F_1	S_7S_c:S_6S_c = 1:1	7:13	1.80	>0.1
F_1-20	(S_7S_c)	self		F_2	S_7S_c:S_cS_c = 1:1	17:20	0.24	>0.5
F_1-20	(S_7S_c)	×	P_1 (LA2163, S_6S_7)	BC_1-1	S_6S_7:S_6S_c = 1:1	10:18	2.28	>0.1
P_1	(LA2163, S_6S_7)	×	F_1-20 (S_7S_c)	BC_1-2	S_7S_c:S_6S_c = 1:1	ND[a]		

[a] S-genotype not assigned, but all 40 plants were self-compatible

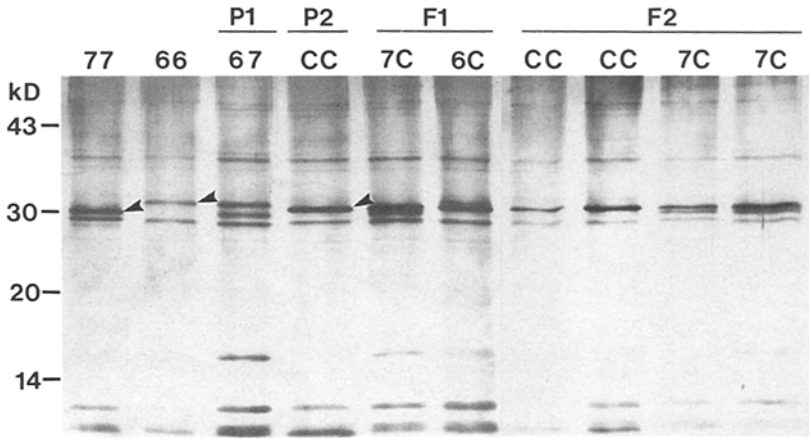

Fig. 2 SDS-polyacrylamide-gel electrophoresis of style extracts from *L.peruvianum* plants with S_7-, S_6- and S_c-alleles in various combinations. Each lane contains style proteins (1.5 μg) from an individual plant and is labelled with the S-genotype assigned by breeding behaviour. The proteins associated with the S_7-, S_6- and S_c-alleles are designated by *arrows*. The gel was silver stained and molecular mass-markers (LKB low molecular mass) are in kDa.

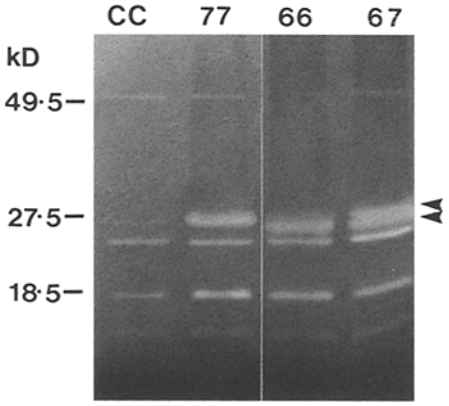

Fig. 3 RNase-activity gel of style extracts from S_7S_7, S_6S_6 and S_cS_c homozygous and the S_6S_7 heterozygous plants. Each lane contains style proteins (30 μg) from a single plant and is labelled with the S-genotype assigned by breeding behaviour. Four ribonucleases with Mrs of about 16, 19, 25 and 56 kDa are common to all genotypes. Plants bearing S_7- and S_6-alleles have an extra band (*arrowed*) of ribonuclease activity of about 28 kDa and 27 kDa respectively

plants contained a major RNase that segregated with the S_c-allele.

RNase activity in buffer-soluble extracts from S_7S_7-, S_6S_6- and S_cS_c-styles

Extracts of styles of the S_cS_c genotypes contained significantly-less ribonuclease activity than styles of the S_7S_7 and S_6S_6 genotypes (Table 2). The comparatively low level of RNase activity in the S_cS_c genotype was even lower in the fractions produced by precipitation with ammonium sulphate (50–95% fraction). When the 50–95% $(NH_4)_2SO_4$ fractions were examined by SDS-PAGE (data not shown) the proteins that segregated with the S_6-,S_7- and S_c-genotypes appeared enriched relative to their levels in the buffer-soluble style extracts. The bands of RNase activity associated with S_6- and S_7-genotypes were also present in the 50–95% $(NH_4)_2SO_4$ fraction whereas no bands were associated with the S_c-genotype.

Discussion

In 1982, Rick reported the discovery of the only natural SC accession of *L. peruvianum* (LA2157) and the presence of SI plants (LA2163) growing in the near vicinity of the SC plants (Rick 1982 b). The similar morphology of the leaves and flower trusses (Fig. 1) of the SI and the SC ac-

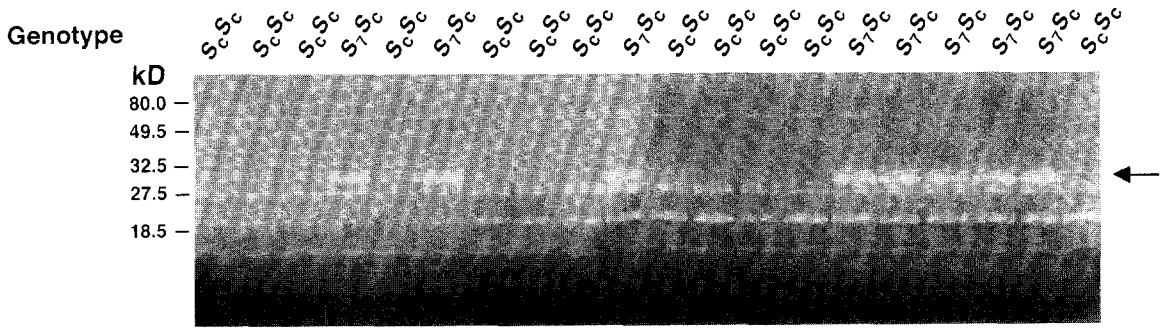

Fig. 4 RNase-activity gel of style extracts from self-compatible plants segregating for the S_7-allele. Each lane contains style proteins from individual F_2 plants derived from selfing an F_1 plant (S_7S_c). All plants with an S_7-allele contain a band of ribonuclease activity (*arrowed*) that is present at much reduced levels in S_cS_c genotypes

Table 2 RNase activity in style extracts from S_7S_7, S_6S_6 and S_cS_c genotypes. Relative to the SI genotypes, buffer-soluble style extracts from the S_cS_c homozygous plants have low levels of RNase that are not detectable after ammonium sulphate fractionation. In contrast, ammonium sulphate fractionation of extracts from SI styles leads to an enrichment of RNase activity. Units = A260 units released per min per mg of protein

Sample	Specific activity U/mg		
	S_6S_6	S_7S_7	S_cS_c
Buffer-soluble extract	4.4	12.9	1.0
95% Ammonium sulphate fraction	6.8	27.2	0

cessions suggests their close relationship since different *L. peruvianum* varieties are often characterized by striking variation in morphology of their leaves and flower trusses (Rick 1963; Taylor 1986). The fact that LA2157 and LA2163 are cross compatible and set viable seed indicates that self-compatibility did not result from a major chromosomal rearrangement. The presence of a functional gametophytic SI system in LA2163 was confirmed as self-pollen did not grow more than one-third to half-way down the style and the arrested tubes had the typical morphology of self-incompatible tubes. Fruit set was never observed after self-pollination of this accession.

The inheritance of self-compatibility in progeny of the SC and SI accessions was studied in F_1, F_2 and backcross progeny. Self-compatibility appeared to be controlled by a single gene because the S-genotype of the progeny obtained in each of the crosses was consistent with the expected progeny if the SC-allele in LA2157 was located at the same locus as the self-incompatibility alleles in the SI parent (Table 1). The S_c-allele did not affect the activity of the S_6- and S_7-alleles when they were present in the same plant since these self-compatible plants could still reject incompatible pollen (see Table 1). From this it was concluded that the S_c-allele was a non-functional allele of the S-locus and not an unlinked modifier of self-incompatibility as has been described previously in *Lycopersicon* (Martin 1968; Rick 1982 a; Rick and Chetelat 1991), *Petunia* (Ai et al. 1991), and *S. tuberosum* (Thompson et al. 1991).

Abundant stylar glycoproteins of about 30 kDa that cosegregate with S-alleles have been described previously in *L.peruvianum* (Mau et al. 1986) and are also present in the accessions used in this study. Proteins of 28.6, 30.4 and 29.7 kDa could be assigned to the S_7-, S_6- and S_c-genotypes, respectively. Co-segregation of these proteins with S-genotypes was confirmed in all members of the F_1 and F_2 populations (57 plants). The protein corresponding to the S_c-allele was produced in approximately equal amounts to the proteins produced by the S_7- and S_6-alleles, indicating that self-compatibility was not the result of altered transcription or translation from the S_c-allele.

The proteins that segregate with the S_7- and S_6-alleles have RNase activity. In contrast, the protein associated with the S_c-allele had no detectable RNase activity and styles of the S_cS_c genotype contained no S-associated RNase bands. The proteins that segregated with the S_6-, S_7- and S_c-alleles were enriched by fractionation with 50–95% ammonium sulphate, which is the first step in the purification protocol for the S-RNases from *Nicotiana alata* (Jahnen et al. 1989). This enrichment was associated with an increase in RNase activity for the S_6- and S_7-alleles but not for the S_c-allele (Table 2). Furthermore, when analysed by RNase-activity gels, the protein co-segregating with the S_c-allele had no detectable activity, although this activity was easily observable for both the S_6- and S_7-alleles (Fig. 3). The absence of RNase activity in the enriched fraction indicates that the S_c-associated protein is not simply an S-RNase with low specific activity but is a protein with similar physical properties which entirely lacks RNase activity. As was noted before, heterozygous plants containing either the S_7- or S_6-allele in combination with the S_c-allele were self-compatible, even though they contained an active style RNase from the S_7- or S_6-allele. In these cases, all fertilization could be attributed to S_c-pollen since these plants retained their ability to reject pollen from either S_7 or S_6 homozygous tester plants. Thus it is possible for a plant with high style-RNase activity to be self-compatible. This has also been observed in *Petunia* (Ai et al. 1991).

One question that has arisen from the discovery that stylar S-glycoproteins are RNases is whether the enzymic function is required for the self-incompatibility response. One hypothesis is that S-RNase is taken up by incompatible pollen tubes in an allele-specific manner leading to degradation of the pollen tube RNA and a cessation of protein synthesis. This would be an effective mechanism to ensure pollen tube death, as pollen tubes do not transcribe rRNA genes and would be unable to replace degraded ribosomes (Mascarenhas 1990; McClure et al. 1990). It is also possible that pollen tubes contain an inhibitor that acts in an allele-specific manner to prevent degradation in compatible pollen tubes. Alternatively, RNase activity of the S-glycoproteins may be unrelated to their function in self-incompatibility (Newbigin et al. 1993). For example, the RNases could have been recruited during evolution for a role in self-incompatibility unrelated to their catalytic activity. This is unlikely because all S-glycoproteins examined to-date have retained their ribonuclease activity (McClure et al.1989; Singh et al. 1991), and all cloned S-alleles contain the conserved histidine and flanking residues (Tsai et al. 1992) that are required for catalytic activity in the related fungal RNase (Kawata et al. 1990). The present study provides further evidence that S-RNases are the determinants of the self-incompatibility phenotype in the styles of solanaceous plants, a result that previously relied upon a number of correlations between the breeding behaviour of S-alleles and different S-RNases and the localization and regulation of expression of S-RNase protein. Furthermore, the S_c-allele indicates that RNase activity is an essential part of self-incompatibility and that loss of RNase activity is associated with self-compatibility.

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Note added in proof

Bernatzky and Miller, working with the same material, have shown independently that S_C is a codominant allele of the S-locus. Bernatzky R, Miller DD Self-incompatibility is codominant in intraspecific hybrids of self-compatible and self-incompatible *Lycopersicon peruvianum* and *L. hirsutum* based on protein and DNA marker analysis. Sexual Plant Reproduction (in press)