Journal of Experimental Botany, Vol. 59, No. 3, pp. 545–552, 2008
doi:10.1093/jxb/erm339 Advance Access publication 10 February, 2008
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Glycosylation of S-RNases may influence pollen rejection thresholds in *Solanum chacoense*

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Received 13 August 2007; Revised 27 November 2007; Accepted 30 November 2007

Abstract

A survey of *Solanum chacoense* plants expressing an authentic S_{11}-RNase transgene identified a line with partial compatibility to S_{11} pollen. By comparing fruit set to the S-RNase levels determined immunologically in single styles, the minimum level of S_{11}-RNase required for full rejection of S_{11} pollen was estimated to be 18 ng per style. The S_{11}-RNase threshold levels are thus considerably lower than those previously reported for the S_{12}-RNase. Interestingly, these two allelic S-RNases differ dramatically in the extent of glycosylation, with the number of glycosylation sites varying from one (S_{11}-RNase) to four (S_{12}-RNase). It is suggested that reduced glycosylation of the S_{11}-RNase may be related to the lower threshold for pollen rejection.

Key words: Gametophytic self-incompatibility, glycosylation, pistil-by-pistil analysis, S-RNase, *Solanum chacoense*, threshold.

Introduction

Self-incompatibility (SI) is a mechanism widespread among flowering plant species that promotes outbreeding by allowing the pistil of a flower to discriminate between genetically related (self) and unrelated (non-self) pollen (de Nettancourt, 1977). In Solanaceae, Rosaceae, and Scrophulariaceae, SI is of the gametophytic type, i.e. the phenotype of the pollen is determined by its haploid genotype and is controlled by a single multigenic S-locus, which includes the highly polymorphic male and female determinants to SI. The S-locus is inherited as a single segregating unit, with variants of the locus termed S-haplotypes and variants of the polymorphic genes residing in the S-locus called alleles (McCubbin and Kao, 2000).

In the families mentioned above, the pistil-expressed *S*-gene product is a secreted glycoprotein with RNase activity termed S-RNase (McClure *et al*., 1989) produced by the cells of the transmitting tract of the style and released in the extracellular matrix, where the pollen tubes grow. S-RNases possess a defined pattern of conserved (C1 through C5) and hypervariable (HVa and HVb) regions, and all S-RNases are glycosylated at a variable number of potential N-glycosylation sites. S-RNases from *N. alata* have been found to contain from one to five sites (Oxley *et al*., 1998) while those from *S. chacoense* contain from one to four sites (Qin *et al*., 2001). The one site conserved in all cases is located in the conserved C2 region in the Solanaceae (Singh and Kao, 1992), whereas in the Rosaceae it is located in the conserved RC4 region (Ishimizu *et al*., 1998). In spite of the fact that S-RNases have been studied for almost 20 years, the biological function of the glycosylation, and in particular that of the conserved glycosylation sites is still unknown. What is known, however, is that the enzymatic removal of the glycan side chains has apparently no effect on the enzyme activity of the native S-RNases *in vitro* (Broothaerts *et al*., 1991). It is also clear that glycosylation is not required for pollen rejection *in vivo*, as when the C2-glycosylation site of the *Petunia hybrida* S_3-RNase was eliminated by site-directed mutagenesis, the mutated S_3-RNase was able to reject S_3 pollen totally (Karunanandaa *et al*., 1994). S-RNases must exert their cytotoxic activity inside the incompatible pollen tubes for pollen rejection to occur, since mutation of essential histidine residues within the active site produces inactive proteins with no RNase activity that are unable to elicit pollen rejection (Huang *et al*., 1994). In *S. chacoense*, immunolocalization studies have revealed that S-RNases enter the cytoplasm of both

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self and non-self pollen tubes (Luu *et al*., 2000), so at least part of the SI mechanism must involve the ability of pollen tubes to block the RNase activity of any non-self S-RNase.

The S-locus product expressed in pollen has been recently identified as an F-box protein, termed either *S*-locus F-box gene (*SLF*) or *S*-haplotype-specific F-box gene (*SFB*) (for details see Kao and Tsukamoto, 2004; McClure and Franklin-Tong, 2006). The usual role assigned to the F-box protein is to mediate protein–protein interactions, which in the context of the E3–ubiquitin ligase complex SCF, helps determine the specificity of the proteins targeted for degradation by the 26S proteasome (Bai *et al*., 1996). Both male and female determinants to SI have now been identified, and the current challenge is to determine how these molecules interact, and to explain how pollen growth is inhibited in an S-haplotype-specific manner. The more traditional models suggest that SLF/SFB interacts with both self and non-self S-RNases inside the pollen tubes. The maintenance of self S-RNase enzyme activity is proposed to occur via S-haplotype-specific interactions between their hypervariable regions and those of their cognate SLF/SFB partners, while all non-self S-RNases are targeted for ubiquitination and subsequent degradation (for details see Kao and Tsukamoto, 2004). A more recent model, based on the observation that S-RNases remain in the vacuole in compatible crosses and are only released into the pollen tube cytoplasm during incompatible pollinations, proposes S-RNase sequestration as a key element in pollen rejection (Goldraij *et al*., 2006). A further model is based on the finding that S-RNases bind more strongly to non-self SLFs than to self SLFs (Hua and Kao, 2006). This suggests that S-RNases enter the pollen tubes cytoplasm and are targeted for degradation by strong binding to the E3 ligase complex during compatible crosses, but remain free to degrade the RNA during incompatible crosses.

A number of factors are known to influence the pollen rejection phenotype, including the presence of other stylar factors, such as the small non-polymorphic HT-B (for details see Cruz-Garcia *et al*., 2005). However, the observation that different amounts of S-RNase can influence the pollen rejection phenotype has been made repeatedly in the literature (Clark *et al*., 1990; Lee *et al*., 1994; Murfett *et al*., 1994; Matton *et al*., 1997, 1999; Qin *et al*., 2001, 2005, 2006; Hiratsuka and Zhang, 2002) and has led to the S-RNase threshold hypothesis. This hypothesis posits that a minimum level of S-RNase is required for pollen rejection, and has been validated for the S_{12}-RNase of *S. chacoense* using genotypes displaying sporadic self-compatibility (Qin *et al*., 2001). The observation that two *S. chacoense* genotypes expressing an S_{12}-RNase, namely 314 and L25, displayed sporadic self-compatibility (Qin *et al*., 2001), followed by a style-by-style analysis of their S_{12}-RNase levels (Qin *et al*., 2006), demonstrated that a variable S_{12} pollen rejection phenotype could be exploited to estimate the threshold, or the minimum level, of the S_{12}-RNase required for pollen rejection. These studies showed that at least 80 ng of the S_{12}-RNase are required for full rejection of pollen from S_{12} homozygous plants. Furthermore, since only 25 ng of S_{12}-RNase is required to reject pollen from an $S_{11}S_{12}$ background, it appears that the RNase threshold differs when pollen donors of different genetic backgrounds are used (Qin *et al*., 2006). Those analyses are extended here to report the determination of S_{11}-RNase threshold by means of style-by-style analysis of a transgenic plant line expressing low levels of the S_{11}-RNase (Matton *et al*., 1997).

Materials and methods

Plant materials and genetic crosses

The plants used in this study include two genetic lines of *Solanum chacoense* called 314 (S_{11}, S_{12}) and L25 (S_{11}, S_{12}) previously noted for their sporadic self-compatibility (Qin *et al*., 2001) and two transgenic plants called T12 and T35, which are a G4 (S_{12}, S_{14}) host line expressing an authentic S_{11}-RNase construct from the chitinase promoter (thus with a stylar haplotype S_{11}, S_{12}, S_{14}) (Matton *et al*., 1997). S_{12} thresholds for 314 pollen in L25 styles and S_{11} thresholds in T12 styles were determined as described (Qin *et al*., 2006).

In order to ensure that only flowers at an identical stage of development were used for both crosses and S-RNase quantification in styles, all open flowers were removed the afternoon preceding crosses and the collection of styles. Genetic crosses were made with freshly collected pollen from plant material grown in the Montreal Botanical Garden greenhouses under natural light conditions, and temperatures were constantly recorded. Pollen viability was estimated by staining with aceto-carmine.

Bacterial expression of S_{11}- and S_{12}-RNases standards

The cDNA fragment encoding S_{11}-RNase was cloned by RT-PCR according to the manufacturer's instruction (Fermentas Inc. MD) using primers 5′-ATGTTTAAATCACTGCTTAC-3′ and 5′-TCAAGGACGAAAAAATATTTT-3′. This fragment, a wild type S_{11}-RNase sequence without the N-terminal signal peptide (22 amino acids), was subcloned into the expression vector pQE30 (Qiagen, Valencia, CA). The sequence was confirmed by sequencing and named pQE30-S_{11}-HisWT. For the S_{12}-RNase, the previously described clone pQE30-S_{12}-HisΔC2, which lacks an essential histidine residue in the active site, was used (Qin *et al*., 2006). Two plasmids were transformed separately into competent M15 cells and protein expression induced by adding IPTG to a final concentration of 1 mM. The cultures were grown at 37 °C or 28 °C 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, and then eluted from gel slices using an Electro-Elutor (Bio-Rad, California, USA) following the manufacturer's protocol. Both RNases were pure, based on Coomassie blue staining after SDS-PAGE and western blot analysis. The concentration of purified S_{11} and S_{12} proteins was determined by the Micro BCA Protein Assay Kit (Pierce Inc., Illinois, USA) following the manufacturer's instructions using BSA as standard. One batch of

each purified protein was used as the standard for all gels to calibrate S-RNases in styles of *S. chacoense*.

Western blots and quantification analysis of S-RNases in individual styles

For western blot analysis, plant styles were collected and immediately frozen in liquid nitrogen. The western blots and quantification analysis on individual styles were performed essentially as described (Qin *et al.*, 2006). Briefly, proteins were extracted from each individual style in 50 µl extraction buffer (0.05 M TRIS pH 8.0, 1 mM DTT, 1 mM EDTA, 0.05 M $CaCl_2$, 1 mM PMSF). The proteins were electrophoresed on SDS–PAGE and transferred to nitrocellulose membrane and stained with 2% (w/v) Ponceau red as a control for a uniform protein loading and transfer; S-RNase measurements from samples with visibly different protein loads were excluded from our analyses. The membrane was blocked by an overnight incubation with 1% w/v BSA fraction V (Sigma) and 2% skimmed milk in TBS-T (TRIS-buffered saline containing 0.05% Tween 20 v/v), 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), rinsed three times with TBS-T, and incubated with 4 µl of 0.5 µCi $mmol^{-1}$ I^{125} of a goat anti-rabbit secondary antibody (Perkin-Elmer) in 10 ml BSA-TBS for 2 h. After washing three times with TBS-T, membranes were exposed to a Phosphor screen for 12–76 h at room temperature and the screen imaged using a PhosphorImager scanner (Amersham Biosciences). The data on the scanner images were quantified using software supplied by the manufacturer. Both antibodies used are specific for their respective substrates and no cross-reaction was detected.

Protein analysis and deglycosylation reaction

Proteins were extracted from each individual style in 50 µl deglycosylation buffer (20 ml TRIS–HCl pH 7.4, 20 mM 2-mercaptoethanol, 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1% Triton X100, 1 mM PMSF, added just before use) (Matton *et al.*, 1997). The N-linked glycan side-chains were enzymatically removed by digestion with Peptide:N-Glycosidase F (PNGase F; New England Biolabs) according to the manufacturer's instructions typically using 500 units PNGase F for every 50 µl style sample. NP-40 was added to a final concentration of 1%, the samples denatured at 100 °C for 10 min and then incubated with l µl (500 units) PNGase F at 37 °C for 3 h. For partial digestion of the glycan side chains, differing amounts (varying from 0.5 to 500 units) of PNGase F were added to the reaction. Reactions were stopped by heating at 100 °C for 5 min with 4× SDS loading buffer. To determine the migration rate and molecular weight of S-RNases, prestained markers (Bio-Rad, CA) were mixed directly with each protein sample. Protein sequences were analysed for *N*-linked glycosylation sites with the NetNGlyc 1.0 Server at http://www.cbs.dtu.dk/services/NetNGlyc/.

Results

The minimum level of S_{11}-RNase required for pollen rejection is very low

In a previous study (Qin *et al.*, 2006), sporadic self compatibility of an S_{12} allele in some genetic backgrounds was exploited to estimate the threshold levels of an S-RNase required for pollen rejection. However, this approach could not be used to estimate the S_{11}-RNase threshold because all naturally occurring S_{11} plants fully reject S_{11} pollen. Indeed, even bud pollinations of these plants conducted up to three days before anthesis failed to produce any $S_{11}S_{11}$ progenies. The lowest amount of S_{11}-RNase measured in these immature buds was 53 ng, suggesting that the threshold for the S_{11}-RNase could be significantly lower than that of the S_{12}-RNase. To address this issue using the same logic used for the S_{12}-RNase, a series of transgenic lines expressing the S_{11}-RNase that had been generated previously were re-examined (Matton *et al.*, 1997). Our attention was drawn to a line (T12) noted for its low levels of mRNA of S_{11}-RNase and partial compatibility (two fruits out of 15 pollinations) (Matton *et al.*, 1997). In order to assess if T12 continued to display partial compatibility towards the S_{11} pollen, a preliminary test was conducted. When crossed with pollen from line 314 (S_{11}, S_{12}), two fruits were obtained out of 10 pollinated flowers. Since the S_{12} pollen from pollen donor line 314 is fully rejected by the endogenous S_{12}-RNase of the host plant used for transgenosis (Matton *et al.*, 1997), any observed compatibility is solely due to the passage of S_{11} pollen. Line T12 is thus partially compatible for the S_{11}-RNase.

The minimum level of S_{11}-RNase required for pollen rejection is pollen-genotype-dependent

Previous work with the S_{12}-RNase indicated that different pollen genotypes respond differently to a given amount of S-RNase (Qin *et al.*, 2006). Thus, T12 was also pollinated with pollen from line L25, which has the same S-constitution (S_{11}, S_{12}) as line 314 but a different genetic background. In an experiment involving 12 crosses with each pollen type, five fruits were obtained using pollen from line 314, whereas no fruits at all were set with L25 pollen. It was concluded from this that the ability to resist rejection by S_{11}-RNase does indeed depend on the genetic background of the pollen, in agreement with previous observations with the S_{12}-RNase (Qin *et al.*, 2006). In addition, since the phenomenon has now been observed both in plants carrying the natural occurring S_{12}-RNase and in transgenics with the S_{11}-RNase, it is likely to be a general feature of SI. This observation also suggests that the threshold for pollen rejection is likely to be lower for L25 than for 314 pollen, since pollen from L25 was completely rejected while pollen from 314 was partially compatible.

In order to test the relationship between the partial compatibility of the T12 line toward S_{11} pollen from line 314 and the levels of S_{11}-RNase in the styles, some styles were harvested for protein analysis at the same time as other flowers were pollinated. As a control plant, styles were also taken from the T35 line that is fully

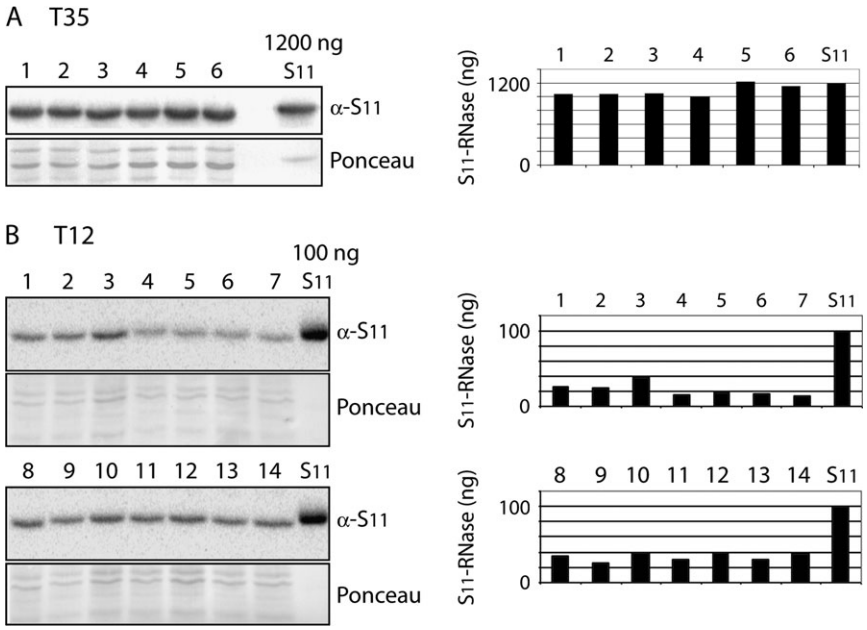

Fig. 1. Low levels of S_{11}-RNase are found in the styles of the partially compatible T12 line. Western blot analysis (left panels) using an anti-S_{11}-RNase were visualized using a PhosphorImager in order to quantify S-RNase levels in protein extracts of individual styles from the fully incompatible T35 (A) and the partially compatible T12 (B) lines. S-RNase levels (right panels) were calculated from the Western using an authentic S_{11}-RNase standard [note that different concentrations were used in (A) and (B) to accommodate the different stylar S-RNase levels].

incompatible with S_{11} pollen (Matton *et al*., 1997). The levels of S_{11}-RNase in line T35 were uniformly high (Fig. 1A) and more than 30-fold higher than those measured in the partially compatible line T12 (Fig. 1B). It is concluded from this that the partial compatibility of the T12 line is correlated with insufficient accumulation of S_{11}-RNase in the styles.

To estimate the minimum amount of stylar S-RNase required for pollen rejection, the range of S_{11}-RNase values in Fig. 1B were redrawn in ascending order (Fig. 2A). In this representation, a vertical dotted line is placed at a position where the number of plants with low S-RNase levels (to the left of the line) roughly corresponds to the percentage of fruits formed using plants pollinated in parallel with the protein analysis (three fruits from 16 pollinations). The minimum amount of S_{11}-RNase needed for pollen rejection is thus estimated as ~18 ng from this analysis. To test the validity of this estimate, the entire experiment was repeated three additional times (Fig. 2B–D). In each case, the number of styles with less than 18 ng S_{11}-RNase is in good agreement with the pollen rejection phenotype as determined by fruit set with 314 pollen (Table 1). The average S-RNase levels in the four experiments also shows a good correlation to the degree of incompatibility (Fig. 2E). Interestingly, as was found for the S_{12}-RNase, pollen genotype affects the threshold estimates. For example, the lowest value of S_{11}-RNase that was detected during pollinations with L25 pollen (where no fruits were set) was 11 ng per style, indicating that this value still lies above the threshold level for this pollen genotype (Table 2).

The glycosylation state of S_{11} and S_{12}-RNases differs

One intriguing difference between the S_{11} and S_{12}-RNases lies in their apparent size on SDS–PAGE (Fig. 3A). Indeed, while the molecular weight of the mature S_{11} and S_{12}-RNases predicted from their cDNA sequences are very similar (22.6 kDa and 22.7 kDa, respectively), the proteins migrate with quite different apparent molecular weights (27 and 36 kDa, respectively). This difference results principally from differential glycosylation, as while complete removal of the sugar side chains with PNGase increases the mobility of both proteins, the difference in size between treated and untreated protein is greater for the S_{12}-RNase than for the S_{11}-RNase. Indeed, assuming a roughly 2 kDa size difference per sugar side chain, the two S_{12}-RNase bands could be interpreted as glycosylation at either three or all four different sites predicted from the sequence. To test this, partial digestion of the glycan side chains was performed using decreasing dilutions of PNGase. By this method, the presence of four different glycosylated forms can be observed depending on the PNGase concentration used (Fig. 3B). These forms agree perfectly with the four glycosylation sites predicted from the primary structure (Fig. 3C), and confirm that each sugar group contributes ~2 kDa to the protein apparent MW (Fig. 3D).

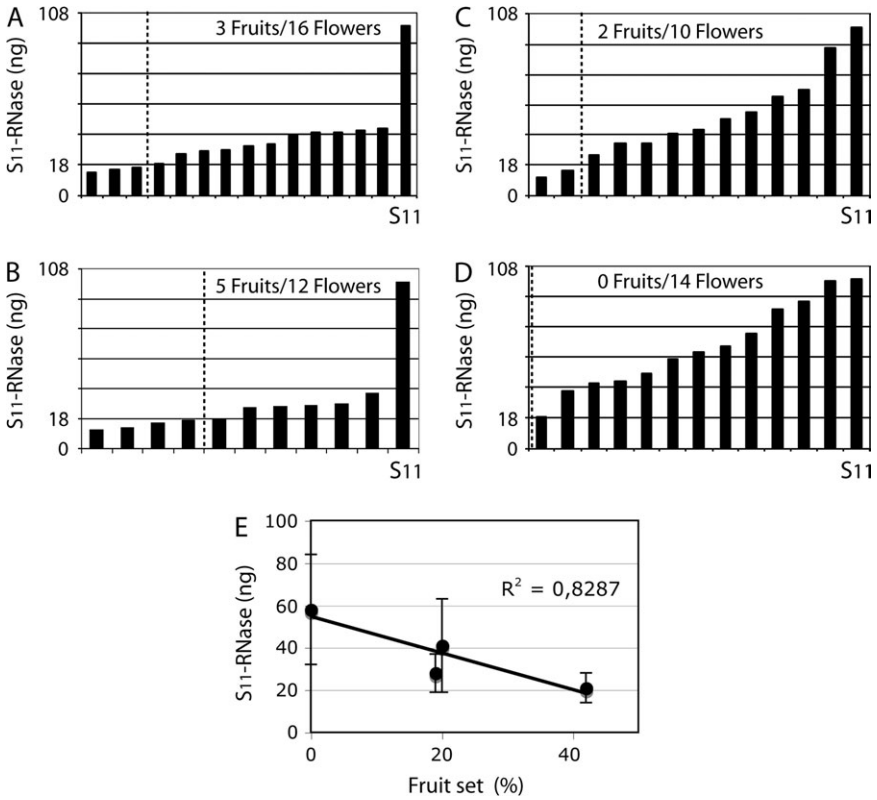

Fig. 2. Pollen rejection requires more that 18 ng S_{11}-RNase per style. S-RNase levels measured in individual styles of T12 plants were ranked in increasing protein content for four different experiments (A–D). For each experiment, the incompatibility phenotype of the same plant was assessed as the number of fruits formed per flower pollinated, and the proportion of plants expected to insufficient levels of S-RNase for pollen rejection indicated by the number of plants to the left of the dotted vertical line. (E) S_{11}-RNase (mean ng ±SD) plotted as a function of degree of incompatibility as determined from fruit set for each of four experiments. The line was drawn by linear regression.

Table 1. *Fruit set for crosses of transgenic lines T12 and T35 with pollen of genotype 314 ($S_{11}S_{12}$)*

Experiment	No.fruits/No.flowers pollinated	
	T12	T35
1	3/16	0/16
2	5/12	0/12
3	0/14	0/14
4	2/10	0/10

Discussion

Our results using the transgenic line T12 with its partial rejection of S_{11} pollen have allowed the threshold for S_{11}-RNase to be determined for two pollen types. These results clearly show that different S-RNases can have different thresholds for pollen rejection as, for pollen from a given donor genotype, there is a marked difference in the thresholds for the S_{11}- and the S_{12}-RNase. For example, rejection of pollen from line 314 (S_{11}, S_{12}) requires ~40 ng of S_{12}-RNase but only ~18 ng of S_{11}-RNase (Table 2). It is also clear that, for a given S-RNase, there is a difference in the ability of the pollen from plants of different genetic backgrounds to resist the cytotoxic effects of the RNase (Table 2). For example, while 18 ng of S_{11}-RNase is required to reject pollen from line 314, less than 11 ng of S_{11}-RNase appear sufficient to reject pollen fully from line L25. It is interesting, however, that the differences in pollen type appear to result in similar threshold differences for both S_{11}- and S_{12}-RNases. One such factor could be a general S-RNase inhibitor capable of binding any S-RNase as proposed previously (Luu *et al.*, 2001) or, according to a more recent model for S-RNase-based SI, an inhibitor blocking the action of other factors playing an essential role in SI (McClure and Franklin-Tong, 2006).

It is tempting to attribute the different thresholds of S_{11}- and S_{12}-RNases required for pollen rejection to differential glycosylation. Clearly, the S_{12}-RNase is more extensively glycosylated than the S_{11}-RNase, as PNGase treatment decreases the apparent molecular weight of the S_{11}-RNase by ~2 kDa, versus the ~9 kDa lost during deglycosylation of the S_{12}-RNase. This difference reflects the larger number of glycan side chains in the S_{12}-RNase. It is possible that a larger, more heavily glycosylated S-RNase may experience increased difficulty in penetrating into the pollen tube cytoplasm. For *S. chacoense*, it has

Table 2. *Pollen genotype effects on thresholds*

Style	No. fruits/No.flowers pollinated		Estimated S_{11} threshold		Estimated S_{12} threshold	
	314	L25	314	L25	314	L25
L25 (86±55 ng S_{12}-RNase)[a]	18/66[a]	5/68			~40 ng	~25 ng[a]
T12 (37±22 ng S_{11}-RNase)	5/12	0/12	~18 ng	<11 ng		

[a] From Qin *et al.* (2006).

previously been shown by immunoelectron microscopy that the S_{11}-RNase accumulates inside pollen tubes to levels roughly 5-fold higher than those found in the transmitting tract (Luu *et al.*, 2000). This hypothesis predicts that the S_{12}-RNase would accumulate at lower levels in pollen tubes, and it would be interesting to test our anti-S_{12}-RNase antibody to see if signal could be detected on cell sections.

Interestingly, recent microscopic observations using *Nicotiana alata* indicate that S-RNases accumulate in the pollen tube vacuole and are released into the cytoplasm as an all-or-nothing response to the presence of a cognate pollen partner to the SI system (Goldraij *et al.*, 2006). Thus, while this model still assumes that cytotoxic effects of the S-RNase cause pollen rejection, differential release of S-RNases from the vacuole into the cytoplasm is unlikely to be the cause of the different thresholds. Indeed, it is likely that all S-RNases found in the vacuole would be released concurrently from vacuoles where the HT-B modifier protein had been stabilized (by as yet undefined components) during an incompatible cross. To date, it is not known if the presence of additional oligosaccharide side chains may interact with modifier proteins such as the small asparagine-rich HT-B (McClure *et al.*, 1999) or the large 120 kDa glycoprotein (Hancock *et al.*, 2005), although the 120 kDa protein has been shown to bind S-RNase *in vitro* (Cruz-Garcia *et al.*, 2005).

In this study, the choice was made to analyse S-RNase levels as they can be quantitatively measured style-by-style, and it is clear from our results that the threshold amount of S-RNase required for pollen rejection is lower for the S_{11}- than for the S_{12}-RNase. However, our proposal that the extent of glycosylation influences the S-RNase threshold does have a number of important caveats. First, the specific activity of the S_{11}- and S_{12}-RNases has not been directly measured and may differ between the two. Different specific activites have been shown for S-RNases in *Petunia* (Broothaerts *et al.*, 1991; Karunanandaa *et al.*, 1994) and *Nicotiana* (McClure *et al.*, 1989), although nothing is yet known for S-RNases in *S. chacoense*. Interestingly, as some of these studies also show that deglycosylation does not alter the specific activity of several different S-RNases (Broothaerts *et al.*, 1991), it seems reasonable to conclude that the extensive glycosylation of the S_{12}-RNase would not appreciably reduce its enzyme activity. Second, it is noted that the transgenic plants expressing the S_{11}-RNase represent a genetic background in which the S_{12}-RNase is fully incompatible, and for which the threshold thus cannot be ascertained. It is therefore a formal possibility that in this genetic background, the activity of modifier proteins such as HT-B and the 120 kDa protein may be different. Indeed, the role played by HT-B or the 120 kDa protein has not yet been characterized in *S. chacoense*. SI is a complex system, and until additional molecular probes become available, many of the complexities may pass unnoticed unless revealed by exceptional genotypes. The two sporadically compatible plants which allowed determination not only of the S_{12}-RNase threshold but also the previously unsuspected influence of the pollen genetic background on the threshold represent an excellent example of this (Qin *et al.*, 2006).

Finally, one of the most surprising aspects of the low S_{11}-RNase threshold shown here is that the 1000 ng S_{11}-RNase in the styles can exceed the required threshold by over 50 times. This vast excess of S-RNase may act as a reinforcing mechanism of the pollen rejection phenotype, and could help explain why self-compatible genotypes do not seem to accumulate in natural populations (Richman and Kohn, 1996). It would also explain the observation that S-RNase-based SI characteristically appears as a qualitative rather than a quantitative genetic trait, as an excess of S-RNase would ensure that the switch-like behaviour of the SI response at threshold levels would never be observed. At present, the generalization of this type of S-RNase excess in natural populations is not known. Clearly, much more work is required to understand fully the intricacy of the SI system.

Acknowledgements

We are grateful to G Teodorescu for plant care. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (MC).

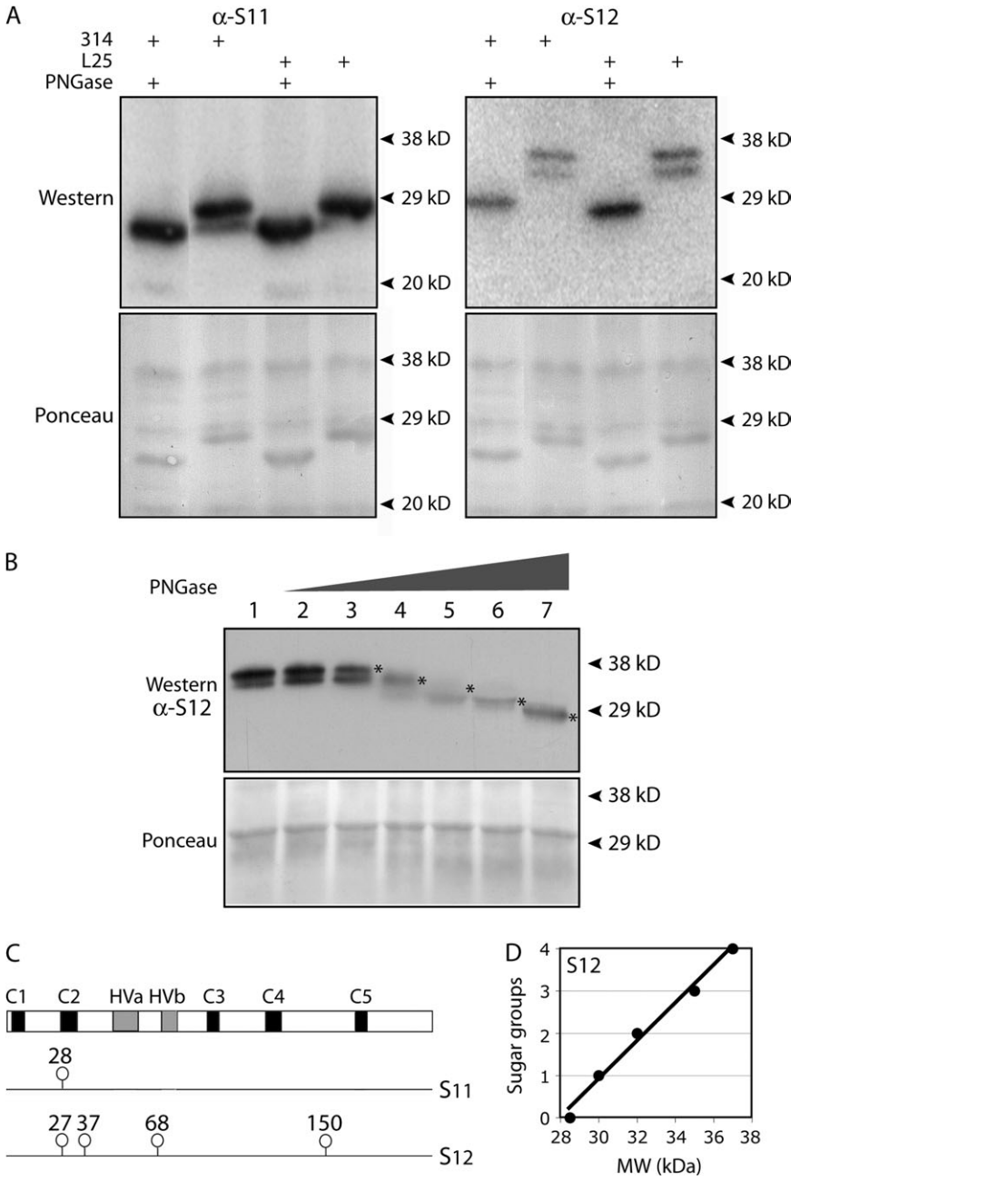

Fig. 3. The S_{11}-RNase is poorly glycosylated compared to the S_{12}-RNase. (A) Western blot of protein extracts from the S_{11}, S_{12} lines 314 and L25 were tested using either anti-S_{11}-RNase (left) and anti-S_{12}-RNase (right) to determine the extent of glycosylation. Part of each extract was treated with PNGase to deglycosylate the S-RNase fully. The three molecular markers indicated by arrowheads were added to all samples before electrophoresis. (B) Differing amounts of PNGase (lane 1, 0 units; lane 2, 0.5 units; lane 3, 2.5 units; lane 4, 5 units; lane 5, 25 units; lane 6, 50 units; lane 7, 500 units) were incubated for 1 h as in (A). Asterisks mark the position of the five different-sized classes of S_{12}-RNase detected. (C) Schematic illustration of the predicted glycosylation sites in the S_{11}- and S_{12}-RNases (open circles). (D) The differing number of predicted glycan side chains is proportional to the apparent molecular weight for the different bands in the S_{12}-RNases.

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