A new dual-specific incompatibility allele revealed by absence of glycosylation in the conserved C2 site of a Solanum chacoense S-RNase

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Abstract

The stylar determinant of gametophytic self-incompatibility (GSI) in Solanaceae, Rosaceae, and Plantaginaceae is an S-RNase encoded by a multiallelic S-locus. The primary structure of S-RNases shows five conserved (C) and two hypervariable (HV) regions, the latter forming a domain implicated in S-haplotype-specific recognition of the pollen determinant to SI. All S-RNases are glycosylated at a conserved site in the C2 region, although previous studies have shown that N-linked glycans at this position are not required for S-haplotype-specific recognition and pollen rejection. Here the incompatibility phenotype of three constructs derived from an originally monoglycosylated S11-RNase of Solanum chacoense, that were designed to explore the role of the HV domain in determining pollen recognition and the role of the N-linked glycan in the C2 region, is reported. In one series of experiments, a second glycosylation site was introduced in the HVa region to test for inhibition of pollen-specific recognition. This modification does not impede pollen rejection, although analysis shows incomplete glycosylation at the new site in the HVa region. A second construct, designed to permit complete glycosylation at the HVa site by suppression of the conserved site in the C2 region, did increase the degree of site occupancy, but, again, glycosylation was incomplete. Plants expressing this construct rejected S11 pollen and, surprisingly, also rejected S13 pollen, thus displaying an unusual dual specificity phenotype. This construct differs from the first by the absence of the conserved C2 glycosylation site, and thus the dual specificity is observed only in the absence of the C2 glycan. A third construct, completely lacking glycosylation sites, conferred an ability to reject only S11 pollen, disproving the hypothesis that lack of a conserved glycan would confer a universal pollen rejection phenotype to the plant.

Key words: Allelic recognition, gametophytic self-incompatibility, glycosylation, site-directed mutagenesis, S-RNase, Solanum chacoense.

Introduction

Self-incompatibility (SI) is a genetic mechanism widespread among flowering plants that allows the pistil of a flower to discriminate between genetically related (self) and unrelated (non-self) pollen (de Nettancourt, 2001). In the Solanaceae, Rosaceae, and Plantaginaceae, the pollen phenotype is determined by its haploid genotype (gametophytic SI, or GSI), and is controlled by a multigenic S-locus, inherited as a single segregating unit, which contains polymorphic male and female determinants to SI. Variants of the S-locus are termed S-haplotypes, whereas variants in the polymorphic genes of the S-locus are called alleles (McCubbin and Kao, 2000). In the families mentioned above, the pistil-expressed S-gene product is an extremely polymorphic glycoprotein with RNase activity termed S-RNase (McClure et al., 1989). The catalytic activity of S-RNases is essential for pollen rejection (Huang et al., 1994; Royo et al., 1994) and this cytotoxic activity must thus be exerted inside the incompatible pollen tubes. However, since S-RNases have been shown by immunolocalization to...
enter pollen tubes in a non-S-haplotype-specific manner (Luu et al., 2000; Goldraij et al., 2006), at least part of the SI mechanism must involve the ability of compatible pollen tubes to block the RNase activity of any non-self S-RNase. This blockage could occur by inhibition of the RNase activity (Kao and McCubbin, 1996; Luu et al., 2001; Sims and Ordanic, 2001), by degradation of the S-RNase (Qiao et al., 2004; Hua and Kao, 2006, 2008; Hua et al., 2008; Meng et al., 2011a), or by blocking access to the RNA substrates in the pollen tube cytosol (McCure, 2008; McClure et al., 2011).

Sequence comparisons of S-RNases in the Solanaceae have revealed a pattern of highly five conserved (C1–C5) and two hypervariable (HV) regions (Parry, 1997). The conserved regions C1, C4, and C5 appear to be involved in stabilizing the three-dimensional structure of the S-RNase (Ioerger et al., 1991; Qin et al., 1998), whereas the conserved regions C2 and C3 each contain a histidine residue essential for RNase activity (Green, 1994). The two HV regions (HVa and HVb) are thought to play a key role in S-haplotype specificity (Kao and McCubbin, 1996; Matton et al., 1997, 1999; Zurek et al., 1997), although other motifs of the protein can contribute to allele specificity (Verica et al., 1998). In the Rosaceae, domains outside the HV region have been shown to be key in determining allelic specificity, as S12- and S13-RNases in Prunus (Rosaceae) are identical in their HV region yet have distinct incompatibility phenotypes (Wunsch and Hormaza, 2004), and similar observations have been made in Pirus (Zisovich et al., 2004). The HV region thus appears not to be the sole determinant for specific pollen rejection.

Curiously, all S-RNases known to date possess potential N-glycosylation sites, the role of which, if any, is still unknown (Meng et al., 2011b). For example, S-RNases from Nicotiana alata have from one to five sites (Oxley et al., 1996, 1998), while those from Solanum chacoense contain from one to four sites (Qin et al., 2001; Liu et al., 2008). There can be differences in the type and structure of the side chains of sugars, termed microheterogeneity, attached to the same S-RNase (Woodward et al., 1992; Oxley and Bacic, 1995) and, in S-RNases with multiple sites, some may remain unoccupied (Parry, 1997). In the Solanaceae, a single glycosylation site, at the N-terminus of the C2 region, is conserved in practically all cases (Singh and Kao, 1992) [with the exception of the S12-RNase from Petunia hybridra whose single glycosylation site is located near the C-terminus of the protein (Clark et al., 1990)]. In the Rosaceae, the conserved glycosylation site is located in the fourth conserved region 4 (RC4) (Ishimizu et al., 1998).

The role of the sugar moieties may be that generally ascribed to secreted proteins, such as facilitation of protein secretion or increased solubility in the extracellular matrix (McCure, 2009). However, enzymatic removal of the glycans side chains has no effect on RNase activity of the protein (Broothaerts et al., 1991), and removal of the C2-glycosylation site of the P. hybridra S11-RNase by site-directed mutagenesis did not alter the ability fully to reject S1 pollen (Karunanandaa et al., 1994). Alternatively, it is possible that the sugar groups may play a more vital role in the SI response. In the case of the Petunia S13-RNase without a glycosylation site, the rejection of other pollen haplotypes was not investigated, and it has been suggested that these transgenic plants might have acquired the ability to reject any pollen type (Oxley et al., 1996). This possibility is intriguing as the S-locus product expressed in pollen (pollen-S determinant) has been identified as an F-box protein (Lai et al., 2002), and N-linked glycans can be a specific target of F-box proteins (Yoshida, 2007). It is also an interesting coincidence that S-like RNases, not involved in SI, are not glycosylated (Green, 1994).

In order to explore the role of glycosylation in the SI response of S. chacoense, two constructs, both with a new N-glycosylation site inside the HVa region of the S11-RNase, were generated. In one, the original glycosylation site in the C2 region (Saba-El-Leil et al., 1994) was maintained, whereas in the other it was removed. Interestingly, the monoglycosylated transgenics harbouring only a glycosylation site in the HVa region were found to reject S13 pollen in addition to S11 pollen. Western analyses revealed the presence of two size classes of protein, suggesting that the non-glycosylated form may reject the S11 pollen while the glycosylated form may reject S13 pollen. In order to confirm that the non-glycosylated form would reject S13 pollen, the conserved glycosylation site in the C2 region of the native S11-RNase was eliminated, validating its ability to reject S13 pollen. This construct also allowed a test of the hypothesis that an S-RNase lacking N-linked glycans might show a universal pollen rejection phenotype.

Materials and methods

Plant material

The self-incompatible diploid genotypes (2n = 2x = 24) of S. chacoense used in the present study include the previously described genetic lines PI 230582 (S11S13) (Rivard et al., 1989), L25 (S12S13) (Qin et al., 2001), G4 (S12S12) (Van Sint Jan et al., 1996), a tetraploid line (S11S11 S12S12) (Luu et al., 2001), and PI 458312 (S13S13) (Liu et al., 2009).

Mutagenesis of the S11-RNase glycosylation sites and plant transformation

A previously described construct (Qin et al., 2005) containing the wild-type S11-RNase (hereafter identified as GlcC2) to indicate that it contains only the endogenous N-glycosylation site located in the C2 conserved region) under control of the chitinase promoter (Harikrishna et al., 1996) in the pBluescript II phagemid cloning vector (Agilent Technologies, Santa Clara, CA, USA) was used as a PCR template for site-directed mutagenesis. All PCRs were carried out using either Taq DNA polymerase (Promega Corporation, Madison, WI, USA) or Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), according to the manufacturers’ instructions.

To create the construct with an additional N-glycosylation site at the HVa region of the S11-RNase (hereafter identified as GlcC2HVa), the AAC codon for lysine in the sequence KLTNYYFSD was changed into a AAT codon for asparagine (K72N) using PCR template for site-directed mutagenesis. The self-incompatible diploid genotypes (2n = 2x = 24) of S. chacoense used in the present study include the previously described genetic lines PI 230582 (S11S13) (Rivard et al., 1989), L25 (S12S13) (Qin et al., 2001), G4 (S12S12) (Van Sint Jan et al., 1996), a tetraploid line (S11S11 S12S12) (Luu et al., 2001), and PI 458312 (S13S13) (Liu et al., 2009).

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The PCR overlap mutagenic primers were GlcC2/HVa A (5’-CGAATGTTCAAAACAATTCAGTCGCTCGG-3’) and GlcC2/HVa B (5’-CCGGTAGAATTGTTTGGGAATGCG-3’), while the two flanking primers in the vector, forward-HindIII 5’-5’-GCGGCGCGTTCACGCTACCATATTCCTC-3’ and reverse-EcoR-I stop (5’-CTCTGATCAAGCAGATTTACATATGCACGATGTA-3’) contained endonuclease restriction sites to facilitate further cloning steps. Both GlcC2/HVa and S11GlcC2/HVa constructs were ligated into pBluescript II using restriction endonucleases HindIII and EcoRI (Promega), and subcloned into the pBIN19 binary plant transformation vector (Clontech, Palo Alto, CA, USA) using the same enzymes.

To create the construct lacking the glycosylation motif in the C2 region (hereafter identified as NoGlc), site-directed mutagenesis by PCR overlap extension (Higuchi et al., 1988) was used to change the AAC codon for asparagine at position 50 of S11-RAse to a CAA codon (N50Q). Mutagenic primers NoGlc A (5’-CAACAGAATGGCTCTCAAAAA CAATTTCACGTTCCGTC-3’) and NoGlc B (5’-AGGCGGTA ATCGTAAATTGTTTGGAAATCGTCCGTCG-3’) were used in combination with flanking primers T7 and T3, respectively, first to generate separate mutated overlapping PCR fragments. These fragments were purified and mixed, and then used with primers T7 and T3 to generate a full-length NoGlc fragment. That fragment was digested using restriction endonucleases XbaI and SalI (Promega), cloned into pBluescript II, then subcloned into the pBIN19 binary plant transformation vector (Clontech) using the same enzymes.

Sequencing validated all three mutated ChiP-S 11-RNase constructs after cloning into the pBIN19 transformation vector (Clontech), which were then transformed into Agrobacterium tumefaciens LBA4404. Plants were transformed by the leaf disc method and regenerated as described previously (Matton et al., 1997) using the S. chacoense genotype G4 carrying the S12S14 alleles.

Crosses and pollen observation

Genetic crosses of transgenic plants were performed using pollen freshly collected from various genotypes as described (Qin et al., 2001). Where appropriate, styles were stained with aniline blue 48 h post-pollination and examined by fluorescence microscopy to assess pollen tube growth (Matton et al., 1997).

Western blot analyses and deglycosylation of S-RNases

Stylar proteins were extracted from both transgenic and non-transgenic plants as described (Qin et al., 2001, 2005). The protein concentration of crude extracts from 10 styles of each plant was determined by Bradford protein assay (Bradford, 1976). Deglycosylated protein samples containing 40 µg of total proteins were prepared by digestion with peptide-N-glycosidase F (PNGase F) (New England Biolabs) according to the manufacturer’s instructions. Non-deglycosylated samples were prepared by the same procedure but omitting the PNGase F enzyme.

Samples were analysed by SDS–PAGE with 20 µg of total stylar protein loaded per lane. Proteins were then transferred to Hybond C-Extra nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Protein transfer was visualized after staining the membranes with Ponceau S using an ImageQuant LAS 4000 imaging system (GE Healthcare).

Immunoblots were performed as described (Qin et al., 2005) with a specific primary antibody raised against a 15 amino acid peptide corresponding to the HVa region of the S11-RAse and a commercial hors eradish peroxidase (HRP)-conjugated secondary antibody (Sigma-Aldrich Corporation, St Louis, MO, USA). HRP activity was visualized using the Immobilon Western Chemiluminescent HRP substrate kit (EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer’s instructions and recorded using an ImageQuant LAS 4000 imaging system.

Results

GlcC2/HVa transgenic plants

A previous report of self-compatible Nicotiana sylvestris found that styles expressed high levels of a stylar ‘relief S-RNase’ (Golz et al., 1998). To explain why this species is self-compatible, the authors suggested that an N-glycosylation site inside the hypervariable HVa region might be involved. To test experimentally the hypothesis that a bulky polysaccharide moiety in the HVa region might prevent the pollen S-gene product from interacting with the S-RNase thus leading to compatibility, an S11-RAse with an extra glycosylation site in the HVa region was first produced.

Protein levels corresponding to the GlcC2/HVa S-RNase, assessed by western blots, varied from almost undetectable to levels higher than wild-type S11-RAses (Fig. 1). Genetic crosses assessed the impact of these variable levels of GlcC2/HVa on the incompatibility phenotype. All plants were self-incompatible and fully incompatible with pollen from the untransformed G4 (S12S14) line. In addition, crosses using pollen from PI 230582 (S13S14) were compatible as expected, due to the presence of compatible S13 pollen. The crosses with pollen from line L25 (S13S14) revealed that transgenic plants with low expression levels, such as line 17, had an incomplete S11-rejection phenotype, whereas plants with higher expression levels (lines S13 26, 27, and 35) fully rejected S13 pollen (Fig. 1). Microscopic observations confirmed the complete arrest of S13 pollen tubes inside the styles of plants highly expressing the transgene. The rejection of S13 pollen was surprising, as it had been predicted that the glycosylation site in the HVa region, previously identified as one of the two allelic recognition domains in S11-RAse (Matton et al., 1997), would block binding to this region of the S-RNase. To assess the possibility that the HVa site was not fully occupied, the stylar extracts were analysed before and after treatment with PNGase. Before treatment, two size classes of protein reacting with the anti-S13 antibody could be detected. One size class, more abundant and having the same apparent molecular mass, was interpreted as the diglycosylated form of the protein. The second size class, with a slightly slower mobility (28 kDa), was interpreted as the deglycosylated form of the protein. This suggests that one of the two sites was indeed not fully occupied.

Interestingly, two size classes of protein reacting with the anti-S11 antibody were still present after treatments with PNGase, suggesting that the presence of the sugar group did not interfere with the antibody reaction. It is concluded that incomplete glycosylation of the
HVα glycosylation site could result in an S_{11} pollen rejection phenotype due to an S_{11}-RNase glycosylated only at the C2 region, as this monoglycosylated form is expected to be identical to the wild type. GlcHVα transgenic plants

The inability of the Glc^{C2HVα} plants to glycosylate both sites fully may have resulted from the proximity of the two sites and a difficulty in accessing sufficient amounts of the glycosylating precursor. Another modified S_{11}-RNase, termed Glc^{HVα}, which contains only a single glycosylation site inside the HVα region was therefore produced. The genetic analysis of 41 Glc^{HVα} transgenics revealed that all plants were self-incompatible and incompatible with line G4 (S_{12}S_{14}). When crossed with pollen from line L25 (S_{11}S_{12}), three individuals termed lines GlcHVα-13, -16, and -37, were found to reject S_{11} pollen completely (Fig. 2A). This pollen rejection phenotype correlated with the amounts of the Glc^{HVα}-RNase which was similar to that found in the L25 control plants as detected by western blot analysis (Fig. 2A). Two possibilities could account for the acquisition of this unexpected dual-specific pollen rejection phenotype. In the first, one of the two forms of the protein could have acquired the dual-specific phenotype, perhaps due to the modified amino acid in the HVα region similar to a dual-specific allele previously generated by site-directed mutagenesis in the HVα region of the S_{11}-RNase (Table 1) (Matton et al., 1999). Alternatively, as two S-RNase forms (glycosylated and non-glycosylated) are found in the transgenics, one form might confer the S_{11}-rejection phenotype while the other might confer the S_{13}-rejection phenotype. To assess the possibility that a single form of the Glc^{HVα}-RNase could recognize and reject both S_{11} and S_{13} pollen, these individuals were submitted to the heteroallelic pollen test (Luu et al., 2001) in which the previously described dual-specific allele was found to reject the normally compatible heteroallelic diploid pollen derived from a S_{11}S_{11}S_{13}S_{13} individual (Luu et al., 2001). As shown (Fig. 2A), this behaviour is also evident in Glc^{HVα}-RNase-expressing plants, supporting its identification as a new dual-specific allele.

![Fig. 1.](https://academic.oup.com/jxb/article-abstract/64/7/1995/581564) Plants expressing a diglycosylated S_{11}-RNase have a normal S-phenotype. Glc^{C2HVα} plant lines express an S_{11}-RNase that contains a glycosylation site in the HVα region in addition to the normal and conserved glycosylation site in the C2 region. Western blot analysis of six selected plants shows a range of protein levels detectable using an anti-S_{11}-RNase antibody. The size of the bands in the absence of PNGase (−) is consistent with a mono- and a diglycosylated form, while in the presence of PNGase (+) the sizes are consistent with a non-glycosylated and a monoglycosylated form. L25 plants (S_{11}S_{12}) and the untransformed host G4 (S_{12}S_{14}) are shown as positive and negative controls, respectively. The pollination phenotype of each plant is shown for a representative pollination with S_{11}S_{12} and S_{13}S_{14} tester stocks and is reported as the number of fruits set per pollination.
A new dual-specific incompatibility allele in *Solanum chacoense*

To test the possibility that a non-glycosylated S-RNase would represent a universal rejector, an additional construct, called NoGlc, lacking the original glycosylation motif in the C2 region was generated. The genetic analysis of 81 NoGlc transgenics revealed that all plants were self-incompatible and incompatible with line G4. Western blot analyses performed on these plants showed a variable level of signal. The crosses with pollen from line L25 (**S11S12**) revealed that transgenic plants with low expression levels had an incomplete **S11** rejection phenotype, whereas plants with higher expression levels fully rejected **S11** pollen (Fig. 3). In order to assess whether or not the plants highly expressing the transgene were able to reject other pollen types, they were pollinated with pollen from line PI 230582 (**S11S11S31S13**). All crosses resulted in full compatibility. These plants were also crossed with pollen from an unrelated genotype PI458312 (**S15S16**). The results of the crosses indicated once again full compatibility (Fig. 3). From these results, it is concluded that the lack of glycosylation of the S-RNases does not prevent them from being functional and does not generate an S-RNase resistant to degradation.

**Discussion**

In this study, two aspects of S-RNase glycosylation were assessed. It was first sought to determine if the presence of an N-linked glycan inside the HVa region would affect pollen rejection. Western blot analysis of seven plant lines expressing an **S11**-RNase engineered to contain only a single glycosylation site in the HVa region show a wide range of protein levels (A). L25 plants (**S11S12**) and the untransformed host G4 (**S12S14**) are shown as positive and negative controls, respectively, and the pollination phenotype of each plant is shown for a representative pollination with **S11S12**, **S13S14**, **S15S16**, and **S11S11S31S13** tester stocks. The three highly expressing lines are shown with (+) and without (−) PNGase treatment (B).

**Table 1.** Sequence comparison of the GlcHVα and the previously described dual-specific HVpβ-RNase.

<table>
<thead>
<tr>
<th>S-RNase</th>
<th>HVa sequence</th>
<th>HVb sequence</th>
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<tbody>
<tr>
<td><strong>S11</strong>-RNase</td>
<td>KPKLYNYF</td>
<td>IDQASARK</td>
</tr>
<tr>
<td>GlcHVα</td>
<td>KPNLTNYF</td>
<td>IDQASARK</td>
</tr>
<tr>
<td>HVpβ</td>
<td>KPKLYNYFF</td>
<td>IDQASALK</td>
</tr>
<tr>
<td><strong>S15</strong>-RNase</td>
<td>KPKLYNYFF</td>
<td>IDQASALK</td>
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**Fig. 2.** Plants expressing the GlcHVα-RNase have an unusual dual specificity incompatibility phenotype. Western blot analysis of seven plant lines expressing an **S11**-RNase engineered to contain only a single glycosylation site in the HVa region show a wide range of protein levels (A). L25 plants (**S11S12**) and the untransformed host G4 (**S12S14**) are shown as positive and negative controls, respectively, and the pollination phenotype of each plant is shown for a representative pollination with **S11S12**, **S13S14**, and **S15S16** testers stocks. The three highly expressing lines are shown with (+) and without (−) PNGase treatment (B).

**Fig. 3.** Plants expressing the NoGlc S-RNase have a normal S-phenotype. Western blot analysis of seven plant lines expressing an S-RNase lacking all glycosylation sites shows a range of protein levels. L25 plants are shown as a positive control, and the pollination phenotype of each plant is shown for a representative pollination with **S11S12**, **S13S14**, and **S15S16** tester stocks.
recognition, as suggested by a study using the self-compatible *N. sylvestris* expressing a stylar ‘relic S-RNase’ with an N-glycosylation site inside the HVa region (Golz *et al.*, 1998). Secondly, it was thought to be of interest to determine if a non-glycosylated S-RNase could reject all different pollen types as proposed by Oxley *et al.* (1996, 1998).

Relic S-RNases, found in both self-compatible and self-incompatible species (Lee *et al.*, 1992; Golz *et al.*, 1998; Liang *et al.*, 2003; Roldan *et al.*, 2010), are a particular type of S-like RNases, structurally similar to functional S-RNases and expressed exclusively in the pistil (Roldan *et al.*, 2010). However, in common with S-like RNases, they are not coded by the S-locus and are not associated with SI. The relic S-RNase from *N. sylvestris* displayed levels of S-RNase activity comparable with those found in self-incompatible *Nicottiana* species (Golz *et al.*, 1998), and it was suggested that the bulky polysaccharide moiety in the HVa region might prevent the pollen S-gene product from interacting with the S-RNase, leading to compatibility.

The explanation proposed by Golz *et al.* (1998) predicts that blocking recognition of the HVa region would lead to compatibility, even in styles with high levels of transgene expression. This is an interesting prediction since a new model of the pollen rejection mechanism, termed the collaborative non-self-recognition system (Kubo *et al.*, 2010; Kakui *et al.*, 2011), actually predicts the opposite result. The collaborative non-self-recognition model, based on the finding that the S-locus contains a number of pollen recognition F-box proteins, proposes that one or a combination of these F-box proteins is able to recognize and degrade any S-RNase with the exception of that encoded by the same S-locus. Blocking access to the recognition domain in the HVa region should thus make the S-RNase resistant to degradation, resulting in pollen rejection.

The first attempt to introduce a glycosylation site in the HVa region, the GlcHVαC2 plants, produced five individuals that turned out to reject *S*11 pollen completely while allowing passage of *S*13 pollen. This S-RNase is not a universal rejector. However, an analysis of the S-RNase by western blot revealed that glycosylation at the site in the HVa region was incomplete. This conclusion is based on the finding that the GlcHVa plants express two different forms of the S-RNase with distinct molecular weights (Fig. 1). These two size classes are interpreted as mono- and diglycosylated forms of the enzyme, with the monoglycosylated form being more abundant. The monoglycosylated form is also interpreted as mono- and diglycosylated forms is significantly different before and after PNGase treatment. This was interpreted to mean that part of the monoglycosylated form is sensitive, and part resistant, to PNGase activity. This is not infrequent in plants, as addition of α(1–3)-fucose to the N-acetyl glucosamine attached to the asparagine protects the sugar group against PNGase treatment (Tretter *et al.*, 1991).

Even more surprisingly, however, the three plants that contained sufficient S-RNase to reject *S*11 pollen were also able to reject *S*13 pollen partially. At least in part, this double specificity may relate to the inherent similarity between *S*11 and *S*13 RNases as the two mature protein sequences share 95% sequence identity. Indeed, four of the amino acid differences between the two lie in the HV region, and it was previously reported that mutation of three of these in the *S*11 sequence also allowed rejection of both *S*11 and *S*13 pollen (Matton *et al.*, 1999). However, the C2 region of the previously generated dual-specific S-allele is fully glycosylated and thus differs from the GlcHVα plants in which mutagenesis of the acceptor site in the C2 region blocks glycosylation at this site.

It is proposed that rejection of both *S*11 pollen and *S*13 pollen occurs because the unglycosylated form of the RNase in transgenic GlcHVα plants has acquired a dual specificity incompatibility phenotype. This phenotype could be explained by the self-recognition model (Fig. 4) if the site used to recognize the RNase as an *S*13 haplotype was partially occluded by the sugar group in the C2 region, thus explaining why the GlcHVα cannot reject *S*13 pollen. This idea is supported by the observation that in the Rosaceae S-RNases can be phenotypically different despite having identical sequences in the HV region (Wunsch and Hormaza, 2004; Zisovich *et al.*, 2004). Interestingly, this observation also implies that sugar groups remain on the S-RNase even after entry into the pollen tube cytoplasm.

However, it is important to note that the dual-specific phenotype can also be accommodated by the collaborative non-self-recognition model. In this case, since the incompatibility of an individual haplotype results from the lack of a specific SLF capable of targeting the S-RNase for degradation, glycosylation of the GlcHVα RNase would impede binding by the SLF that normally recognizes and degrades the *S*13-RNase.

The surprising rejection of *S*13 pollen, which occurs at least partially in three of the GlcHVα plants, is reminiscent of a previously characterized dual-specific form of the *S*11-RNase (Matton *et al.*, 1999). The new dual-specific allele shares with the previously described dual-specific allele a modification in the HV region (Table 1) that replaces a positively charged amino acid (lysine in the HVa region of the GlcHVα, and arginine in the HVb region of the HVapb) with an uncharged amino acid (asparagine and leucine for GlcHVα and HVapb, recognition.
respectively). Curiously, the new allele differs from the previous allele in that the dual specificity is only revealed when the S-RNase cannot be glycosylated in the normal C2 region. It is important to stress that the unusual rejection phenotype of S-RNase cannot be glycosylated in the normal C2 region. A hypothetical interaction with the pollen component (dotted line) is shown for recognition by either the Hya or the conserved C2 region. The glycan groups are shown as twigs at the protein surface, in either the hypervariable Hya region or the conserved C2 region. A hypothetical interaction with the pollen component (dotted line) is shown for recognition by either the Hya or the conserved C2 region. The glycan groups are shown as twigs at the protein surface, in either the hypervariable Hya region or the conserved C2 region.

![Fig. 4. Model for the different S-RNases produced by GlcC2/Hya and GlcHya transgenic lines. Each of the transgenic lines produces two S-RNases forms (spheres), differing from one another with regard to glycosylation at the newly introduced site in the Hya region. The glycan groups are shown as twigs at the protein surface, in either the hypervariable Hya region or the conserved C2 region. A hypothetical interaction with the pollen component (dotted line) is shown for recognition by either the Hya or the conserved C2 region.](https://example.com/f4.png)

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References


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