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Carbohydrate Moiety of the *Petunia inflata* S_3 Protein Is Not Required for Self-Incompatibility Interactions between Pollen and Pistil

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For *Petunia inflata* and *Nicotiana alata*, which display gametophytic self-incompatibility, S proteins (the products of the multiallelic *S* gene in the pistil) have been shown to control the pistil's ability to recognize and reject self-pollen. The biochemical mechanism for rejection of self-pollen by S proteins has been shown to involve their ribonuclease activity; however, the molecular basis for self/non-self recognition by S proteins is not yet understood. Here, we addressed whether the glycan chain of the S_3 protein of *P. inflata* is involved in self/non-self recognition by producing a nonglycosylated S_3 protein in transgenic plants and examining the effect of deglycosylation on the ability of the S_3 protein to reject S_3 pollen. The S_3 gene was mutagenized by replacing the codon for Asn-29, which is the only potential N-glycosylation site of the S_3 protein, with a codon for Asp, and the mutant S_3 gene was introduced into *P. inflata* plants of the S_1S_2 genotype. Six transgenic plants that produced a normal level of the nonglycosylated S_3 protein acquired the ability to reject S_3 pollen completely. These results suggest that the carbohydrate moiety of the S_3 protein does not play a role in recognition or rejection of self-pollen and that the *S* allele specificity determinant of the S_3 protein and those S proteins that contain a single glycan chain at the same site as the S_3 protein must reside in the amino acid sequence itself.

INTRODUCTION

Gametophytic self-incompatibility is an intraspecific reproductive barrier that prevents inbreeding and promotes outcrossing in flowering plants (de Nettancourt, 1977). Gametophytic self-incompatibility displayed by several members of the Solanaceae, including *Petunia inflata*, is controlled by a single multiallelic locus called the *S* locus. Matching of the *S* allele carried by the pollen with either of the two *S* alleles carried by the pistil results in inhibition of pollen tube growth in the style. Pistil proteins that cosegregate with *S* alleles have been identified and extensively characterized (for reviews, see Singh and Kao, 1992; Newbigin et al., 1993). These proteins are called S proteins or S RNases because they exhibit ribonuclease activity, a finding first made in several S proteins of *Nicotiana alata* (McClure et al., 1989). The role of S proteins in recognition and rejection of self-pollen has recently been firmly established in *P. inflata* (Lee et al., 1994) and in *N. alata* (Murfett et al., 1994), and the biochemical mechanism for the rejection of self-pollen in *P. inflata* has subsequently been shown to involve the ribonuclease activity of S proteins (Huang et al., 1994).

Currently, it is not known how S proteins distinguish between self-pollen and non-self-pollen during self-incompatibility (SI) interactions. This is mainly because the gene controlling the SI behavior of pollen has not yet been identified. However, irrespective of the nature of the pollen *S* gene, we can expect that it must be as polymorphic as the pistil *S* gene and that both pistil S proteins and the products of pollen *S* alleles must contain *S* allele specificity determinants to allow them to interact in an allele-specific manner to elicit SI responses. Thus, identification of the *S* allele specificity determinant of S proteins should help in designing strategies to identify the products of pollen *S* alleles.

Because S proteins are glycoproteins with N-linked glycans, the *S* allele specificity determinant may lie in the carbohydrate moiety, the amino acid sequence itself, or both (Woodward et al., 1989). In animals, the glycan chains of many glycoproteins have been shown to play important roles in cell–cell recognition (for reviews, see Kobata, 1992; Lis and Sharon, 1993). For example, in mice, the specific recognition between sperm and eggs involves the O-linked glycans of a glycoprotein

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located on the extracellular coat of the egg and a carbohydrate binding protein on the sperm (Wassarman, 1990). In plants, the role of glycans of glycoproteins in cellular recognition is less clear; however, the fact that cell wall oligosaccharides can serve as signal molecules in plant–pathogen interactions, plant development, and plant–symbiont interactions (Ryan and Farmer, 1991) would suggest a possible role for the glycans in cellular recognition.

Thus, attempts have previously been made to examine the role of the carbohydrate moiety of the pistil recognition molecules (presumably S proteins) in SI interactions. Using an in vitro bioassay developed for *P. hybrida*, it was found that germination and tube growth of 60% of self-pollen was inhibited by pistil extracts; however, when lectins were added to the pistil extracts, the percentage of inhibition was reduced to 30% (Sharma and Shivanna, 1983). In follow-up experiments, Sharma et al. (1985) treated stigmas of *P. hybrida* with lectins, either concanavalin A (ConA) or phytohemagglutinin, prior to self-pollinations and found that 20% (in the case of ConA treatment) and 50% (in the case of phytohemagglutinin treatment) of the plants tested set fruits; however, the seed number per fruit (~100) was lower than that obtained from cross-pollinations (~800). These results were taken to mean that the recognition molecules in the pistil contained saccharides and that binding of saccharides with lectins made them ineffective in recognizing self-pollen. However, besides the fact that the effect observed was not all or none, these results are far from conclusive for the following reasons. First, the in vitro pollen germination system cannot faithfully mimic the in vivo milieu encountered by pollen tubes (Jackson and Linskens, 1990; Gray et al., 1991; Singh and Kao, 1992), so the results obtained using this system may not be physiologically relevant. Second, lectins can also bind other glycoproteins present in the pistil, so it was not possible to attribute the inhibitory effect of lectins, observed in vitro and in vivo, to the loss of S protein activity per se.

More recently, the role of the carbohydrate moiety in the ribonuclease activity of *P. inflata* and *P. hybrida* S proteins has also been examined. Enzymatic deglycosylation of four *P. hybrida* S proteins was found not to affect their ribonuclease activity, and this finding led to the suggestion that the carbohydrate moiety of S proteins might be involved in the recognition function during SI interactions (Broothaerts et al., 1991). Also, a nonglycosylated recombinant S_3 protein of *P. inflata* produced in insect cells by using a baculovirus expression system was found to have ribonuclease activity based on an activity gel staining assay (Mu and Kao, 1992).

One direct approach to examine the role of the carbohydrate moiety of S proteins in SI interactions is to study the effect of deglycosylation of S proteins on their function in SI interactions. S proteins whose sequences have been reported contain one to five potential N-glycosylation sites with the consensus sequence Asn-X-Ser/Thr (Tsai et al., 1992; Rivers et al., 1993; Dupres et al., 1994; Royo et al., 1994). Among them, only the site (Asn-Phe-Thr) located in the conserved region C2 is nearly invariant (Tsai et al., 1992); it is present in all except one S protein, PS1B of *P. hybrida* (Clark et al., 1990). This site is also the only potential N-glycosylation site in the S_3 protein of *P. inflata* (Ai et al., 1990).

Here, we transformed S_1S_2 plants of *P. inflata* with a mutagenized S_3 gene that had the codon for Asn-29 of the Asn-Phe-Thr sequence replaced with a codon for Asp and examined whether the production of a nonglycosylated S_3 protein in the pistils of the transgenic plants would confer on them the ability to reject S_3 pollen, as the production of the wild-type glycosylated S_3 protein in transgenic plants has been previously shown to do (Lee et al., 1994).

RESULTS

Transformation of *P. inflata* Plants of the S_1S_2 Genotype with a Mutant S_3 Gene That Had the Codon for Asn-29 Replaced with a Codon for Asp

We previously introduced a ~3.6-kb DNA fragment of the S_3 gene of *P. inflata* into *P. inflata* plants of the S_1S_2 genotype and showed that production of a normal level of the S_3 protein conferred the ability to reject completely S_3 pollen on transgenic plants (Lee et al., 1994). Thus, we chose to use this DNA fragment for engineering a mutant S_3 gene; we replaced the AAC codon for Asn-29 with a GAC codon for Asp (see Methods). Asp was selected because of its structural similarity with Asn so as to minimize alteration in the structure of the mutant protein. In addition, PS1B of *P. hybrida*, which is the only S protein that does not have Asn at this nearly invariant N-glycosylation site, has Asp instead (Clark et al., 1990).

The mutant S_3 gene, designated *S_3(N29D)*, was introduced into *P. inflata* plants of the S_1S_2 genotype via Agrobacterium-mediated transformation (Lee et al., 1994). A total of 163 transgenic plants were obtained.

Examination of Expression Levels of a Mutant S_3 Gene in Pistils of Transgenic Plants

To determine whether the transgene was expressed in the transgenic plants, we first analyzed the total pistil protein of each transgenic plant by SDS-PAGE to search for a new protein not present in plants of the S_1S_2 genotype from which the transgenic plants were derived. The majority of the transgenic plants (142 plants) did not produce any detectable new protein; however, a new protein with an estimated molecular mass of 22 kD was found in 21 transgenic plants. The results for representative transgenic plants and nontransgenic plants of the S_1S_2 and S_3S_3 genotypes are shown in Figure 1. This new protein (the band indicated with an arrow in Figure 1) migrated faster than the wild-type glycosylated S_3 protein (the band indicated by z in Figure 1); its estimated molecular mass is 2.5 kD smaller and is close to that predicted for the S_3 protein based on its amino acid sequence.

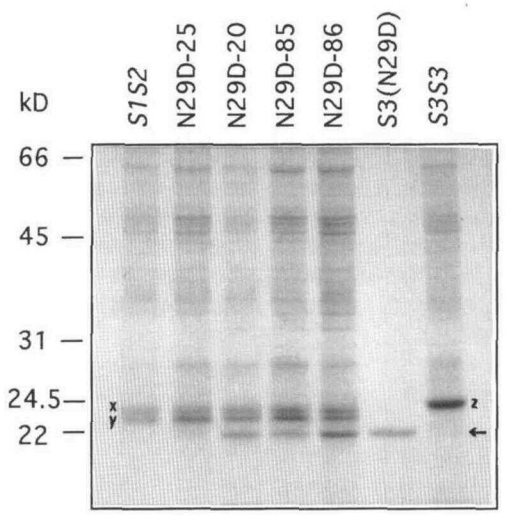

Figure 1. SDS-PAGE of Total Pistil Protein of Representative Transgenic Plants and Nontransgenic Plants.

Total protein, extracted from one pistil of each plant, was electrophoresed in a 12% SDS–polyacrylamide gel, along with the S_3(N29D) protein and molecular mass markers (indicated at left in kilodaltons). S_1S_2 and S_3S_3 are nontransgenic plants; N29D-25, N29D-20, N29D-85, and N29D-86 are four of the transgenic plants obtained in this study; S_3(N29D) denotes the purified mutant S_3 protein expressed from the transgene S_3(N29D) (see Figure 2). Proteins were visualized by Coomassie blue staining. The S_2, S_1, and S_3 proteins are indicated by x, y, and z, respectively, and the S_3(N29D) protein is indicated with an arrow.

To quantify better the level of the 22-kD protein produced in the 21 transgenic plants, total pistil protein of each transgenic plant was analyzed by cation exchange column chromatography. For comparison, total pistil protein of nontransgenic S_1S_2 and S_2S_3 plants as well as of a transgenic plant, N29D-25, that did not produce the 22-kD protein were similarly analyzed. All 21 transgenic plants, in addition to producing endogenous S_1 and S_2 proteins, produced a protein that eluted at approximately the same salt concentration as wild-type S_3 protein; the elution profiles of three of these transgenic plants, N29D-20, N29D-85, and N29D-86, are shown in Figure 2. This protein, shown in the lane labeled S_3(N29D) in Figure 1, had a mobility identical to that of the 22-kD protein on the SDS–polyacrylamide gel (see the following discussion for the definition of the S_3(N29D) protein). Based on the elution profiles of the 21 transgenic plants, the levels of the 22-kD protein produced in the pistils of six of them, N29D-37, N29D-72, N29D-78, N29D-83, N29D-85, and N29D-86, were comparable to the level of the S_3 protein produced in the nontransgenic S_2S_3 plant and GS3-41, a previously obtained transgenic plant (Lee et al., 1994) that expressed the wild-type S_3 transgene and that acquired the ability to reject S_3 pollen (compare the elution profiles of N29D-85 and N29D-86 with those of S_2S_3 and GS3-41 shown in Figure 2). The other 15 transgenic plants were found to produce lesser amounts of the 22-kD protein; the profile of one such transgenic plant, N29D-20, is shown in Figure 2.

The N-terminal sequence of nine amino acids of the 22-kD protein was determined and found to match precisely the previously determined sequence of the wild-type S_3 protein (Ai et al., 1990). Thus, the 22-kD protein was most likely the mutant S_3 protein. Because production of a normal level of S proteins in the pistil is crucial for the pistil to reject self-pollen completely (Lee et al., 1994), we chose the six transgenic plants that produced a normal level of the 22-kD protein for further analysis.

DNA and RNA Gel Blot Analyses of Transgenic Plants

To confirm further that the 22-kD protein is indeed the product of the mutant S_3 gene, S_3(N29D), we performed DNA gel blot analysis with the four transgenic plants, whose total pistil protein profiles are shown in Figure 2, to determine whether they

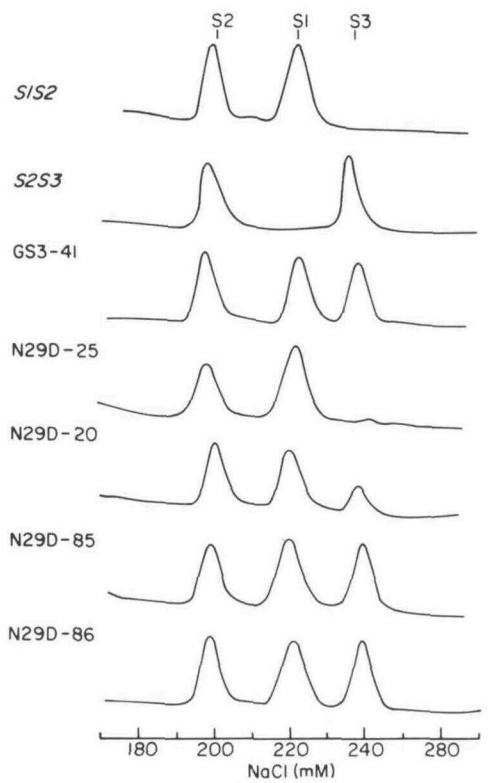

Figure 2. Cation Exchange Chromatographic Profiles of S Proteins.

Total protein, extracted from 10 pistils of each plant, was chromatographed on a Mono-S column. Only the portion of each elution profile containing S proteins is shown. GS3-41 is a previously obtained transgenic plant that expresses a normal level of both the wild-type S_3 transgene and the endogenous S_1 and S_2 genes (Lee et al., 1994). Refer to Figure 1 for the identity of the other plants.

carried the transgene. A blot of EcoRI-digested genomic DNA was hybridized with a radiolabeled probe of the full-length S_3 cDNA (Ai et al., 1990). The results shown in Figure 3 revealed that all four transgenic plants contained a 2.5-kb hybridizing fragment that was also present in a nontransgenic plant of the S_1S_2 genotype. This fragment corresponds to the endogenous S_2 gene that cross-hybridized with the S_3 cDNA as the result of sequence similarity (Lee et al., 1994). Transgenic plants N29D-25, N29D-20, N29D-85, and N29D-86 contained three, one, one, and two additional hybridizing DNA fragments, respectively. Each of these DNA fragments resulted from one cut by EcoRI within the integrated transgene and a second cut outside the transgene in the genome. Different sizes of these fragments indicated different integration sites for each transgene in the chromosomes of the transgenic plants.

We also performed RNA gel blot analysis to determine the mRNA level of the transgene. The blot containing total pistil RNA of nontransgenic S_2S_3 and S_1S_2 plants and of the same four transgenic plants previously described was hybridized with a radiolabeled oligonucleotide probe specific for S_3 RNA. As shown in Figure 4, N29D-85 and N29D-86, which produced a normal level of the 22-kD protein, also produced approximately the same level of *S_3(N29D)* RNA as the S_2S_3 plant produced S_3 RNA; N29D-20, which produced less of the 22-kD protein, produced *S_3(N29D)* RNA at approximately one-fourth the level of S_3 RNA produced in the S_2S_3 plant; N29D-25, which did not produce a detectable level of the 22-kD protein, did not produce a detectable level of *S_3(N29D)* RNA either. Thus, we concluded that the 22-kD protein is indeed the product of the mutant S_3 gene, *S_3(N29D)*, and is hereafter referred to as the S_3(N29D) protein.

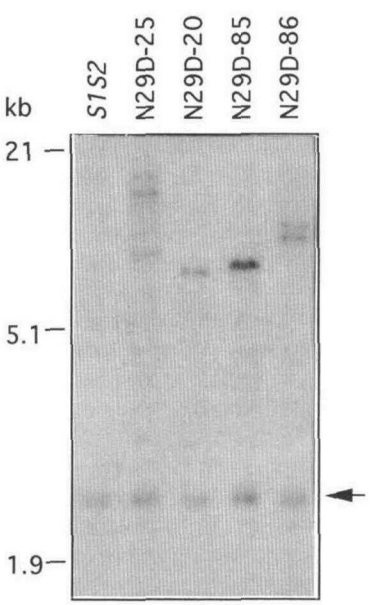

Figure 3. Genomic DNA Gel Blot Analysis.

The autoradiogram shows hybridization of the blot containing EcoRI digests of genomic DNA (10 μg per lane) isolated from the plants indicated using the S_3 cDNA probe. The arrow marks a 2.5-kb DNA fragment that corresponds to the endogenous S_2 gene. The endogenous S_1 gene did not cross-hybridize with the S_3 cDNA probe under the hybridization conditions used. The DNA length markers are indicated at left in kilobases.

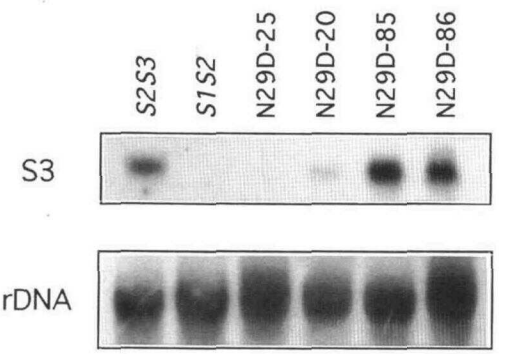

Figure 4. RNA Gel Blot Analysis of *S_3(N29D)* Gene Expression.

The blot contains total pistil RNA (10 μg per lane) isolated from the plants indicated.

(Top) The autoradiogram shows the blot that was hybridized with an oligonucleotide probe specific to sense S_3 RNA (Lee et al., 1994).

(Bottom) The autoradiogram shows rehybridization of the same RNA gel blot after bound radiolabeled probe was removed. The blot was then hybridized with an rDNA probe that encodes the 25S rRNA of *P. inflata*.

Examination of Breeding Behavior of Transgenic Plants

The six transgenic plants that produced a normal level of the S_3(N29D) protein were pollinated with pollen from nontransgenic S_1S_1, S_2S_2, and S_3S_3 plants, and the results are shown in Table 1. As expected, they all completely rejected S_1 and S_2 pollen, as did the wild-type S_1S_2 plant, because these transgenic plants were derived from S_1S_2 plants and produced normal levels of the S_1 and S_2 proteins (Figure 2). More importantly, they also all rejected S_3 pollen completely because no fruits were set. To rule out the possibility that the failure of these transgenic plants to set fruit was due to male or female sterility and not to an SI response, we self-pollinated all six transgenic plants at the immature bud stage, when SI is known to not be expressed (Ai et al., 1990). Large fruits containing seed numbers comparable to those obtained from bud-selfing of wild-type *P. inflata* plants were obtained. Thus, the six transgenic plants indeed had acquired S_3 allele specificity by virtue of the expression of the *S_3(N29D)* gene.

N29D-20, the transgenic plant that produced normal amounts of endogenous S_1 and S_2 proteins but a lesser amount of the S_3(N29D) protein than the six transgenic plants previously

mentioned, was found to reject S_1 and S_2 pollen completely, but not to reject S_3 pollen completely, that is, they produced small fruits (containing an average of 70 seeds per fruit) when pollinated with S_3 pollen (Table 1). This finding reaffirms our previous findings that the level of S proteins produced in the pistil is crucial for the pistil to reject self-pollen completely (Huang et al., 1994; Lee et al., 1994). N29D-25, the transgenic plant that did not produce a detectable amount of the S_3(N29D) protein, did not reject S_3 pollen at all; large fruits were obtained, and each had a seed number (~200) comparable to that obtained from compatible crosses (Table 1).

Biochemical Analysis of the S_3(N29D) Protein

To confirm that the S_3(N29D) protein is indeed nonglycosylated, it was purified from the transgenic N29D-86 plant and subjected to glycan analysis along with the wild-type S_3 protein purified from a nontransgenic S_2S_3 plant. Figure 5A shows these two purified proteins analyzed by SDS-PAGE. Figure 5B shows the results of the glycan assay. The wild-type S_3 protein bound to ConA–peroxidase and appeared brown in color as the result of the product produced by the action of peroxidase on diaminobenzidine (see Methods). However, the S_3(N29D) protein did not cross-react with ConA–peroxidase and hence remained unstained (and in fact was "negatively stained" relative to the background), suggesting that it did not contain a glycan chain.

To determine whether the absence of the glycan chain had an effect on the ribonuclease activity of the S_3(N29D) protein, we measured the specific activity of the purified S_3(N29D) and the three wild-type S proteins, S_1, S_2, and S_3. The results are shown in Table 2. Consistent with the results previously obtained (Singh et al., 1992; Huang et al., 1994), of the three wild-type S proteins, S_2 had the highest specific activity, followed by S_3 and S_1. The specific activity of the S_3(N29D) protein was not significantly lower than that of the wild-type S_3 protein, and it was, in fact, slightly higher than that of the wild-type S_1 protein. This is in sharp contrast to another mutant S_3 protein that we had previously engineered, which had one of the two histidine residues implicated in ribonuclease activity replaced with asparagine; this mutant protein did not have detectable ribonuclease activity (Huang et al., 1994).

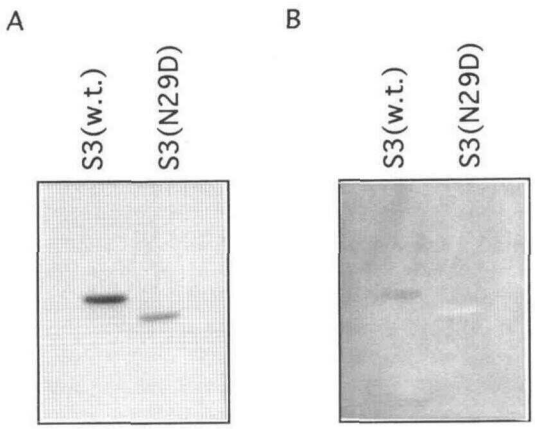

Figure 5. Glycan Analysis of the Wild-Type S_3 Protein and the S_3(N29D) Protein.

(A) Coomassie blue staining of the protein gel blot containing the wild-type S_3 protein (S3(w.t.)) and the S_3(N29D) protein. One hundred pistils each from a nontransgenic S_2S_3 plant and the transgenic N29D-86 plant were used for the purification of the wild-type S_3 protein and the S_3(N29D) protein, respectively, by a two-step procedure (Singh et al., 1991). Each lane of the 12% SDS–polyacrylamide gel contains 1.5 μg of the wild-type S_3 protein or the S_3(N29D) protein.

(B) Detection of the glycan chain in the wild-type S_3 protein and the S_3(N29D) protein. The blot shown in **(A)** was completely destained and used for the glycan assay. The positively stained band in the lane labeled S3(w.t.) represents the glycan-containing protein; the negatively stained band in the lane labeled S3(N29D) represents the non-glycan-containing protein.

Table 1. Results of Pollinations of Transgenic Plants and a Wild-Type Plant with Pollen from Tester Plants

	Pollen Parent		
Pistil Parent	S_1S_1	S_2S_2	S_3S_3
S_1S_2	0/5	0/5	5/5
N29D-37	0/5	0/5	0/15
N29D-72	0/5	0/5	0/10
N29D-78	0/5	0/5	0/10
N29D-83	0/5	0/5	0/15
N29D-85	0/5	0/5	0/15
N29D-86	0/5	0/5	0/15
N29D-20	0/5	0/5	5/5
N29D-25	0/5	0/5	5/5

The results are presented as the number of pollinations that set fruit/total pollinations attempted.

DISCUSSION

Because S proteins alone are sufficient for the pistil to elicit SI responses (Lee et al., 1994; Murfett et al., 1994), they must possess two separate functions: the ability to interact with the as-yet-unidentified pollen *S* allele product to distinguish between self- and non-self-pollen and the ability to inhibit the growth of self-pollen tubes. Our finding that the production of a normal level of the nonglycosylated S_3 protein, S_3(N29D), of *P. inflata* in transgenic plants conferred on them the ability to reject S_3 pollen completely suggests that the nonglycosylated S_3 protein retains both functions of the wild-type glycosylated S_3 protein in SI interactions. Thus, the carbohydrate moiety

Table 2. Ribonuclease Activity of Wild-Type and Nonglycosylated S Proteins of *P. inflata*

Protein	Specificity Activity[a]
S_1 (wild-type)	119.8 (2.4)
S_2 (wild-type)	229.2 (1.6)
S_3 (wild-type)	175.0 (1.8)
S_3(N29D)	138.5 (1.8)

[a] Mean specific activity of three replicates is given as the $A_{260\ nm}$ units of acid solubles released from torula yeast RNA by 1.0 mg of protein in 1 min at 37°C in 10 mM sodium phosphate, pH 7.0. The standard error of the mean is given in parentheses.

of the S_3 protein does not play a role in self/non-self recognition or in rejection of self-pollen.

That the carbohydrate moiety of the S_3 protein is not required for its ribonuclease activity is consistent with our previous finding based on an activity gel staining assay. We determined that the nonglycosylated S_3 protein produced in insect cells using a baculovirus expression system retains its ribonuclease activity (Mu and Kao, 1992). This result is also consistent with the finding by Broothaerts et al. (1991) that enzymatic deglycosylation does not affect the ribonuclease activity of *P. hybrida* S proteins. Because we have previously shown that the ribonuclease activity of the S_3 protein is essential for its function in rejecting self-pollen (Huang et al., 1994), the retention of ribonuclease activity in the S_3(N29D) protein is consistent with its having a normal function in SI interactions.

That the carbohydrate moiety of the S_3 protein is not required for the recognition function suggests that the *S* allele specificity determinant of the S_3 protein must reside in the amino acid sequence and not in the glycan chain of the protein. This conclusion is also most likely valid for a number of other solanaceous S proteins that, like the S_3 protein of *P. inflata*, contain only one potential N-glycosylation site located in the same C2 region of the protein (Tsai et al., 1992). These include the S_1 and S_2 proteins of *P. inflata* (Ai et al., 1990). However, for those S proteins that contain more than one potential N-glycosylation site (one of which has been shown to contain four N-linked glycans; see Woodward et al., 1989), it remains to be determined whether the glycan chains attached to other sites of these S proteins play a role in either the stability or the function of the protein.

It is perhaps not surprising that the determinant of *S* allele specificity of S proteins lies in the amino acid sequence, at least for those that contain a single glycan chain, given the high degree of amino acid sequence diversity among them (Tsai et al., 1992). Differences in the amino acid sequences would be expected to be sufficient to distinguish between different S proteins. One important implication of this finding is that the approach of domain swapping coupled with site-directed mutagenesis can be used to determine the regions and specific amino acids of S proteins that are responsible for *S* allele specificity. For example, various chimeric *S* genes can be constructed from S_1 and S_3 genes of *P. inflata* and introduced into S_2S_2 plants. By determining whether transgenic plants reject S_1 pollen, S_3 pollen, or neither, one can identify the regions that are necessary and/or sufficient for *S* allele specificity. Site-directed mutagenesis can then be performed to pinpoint the specific residues that constitute the *S* allele specificity determinant.

Finally, the demonstration that the nonglycosylated S_3 protein functions as well as the wild-type glycosylated S_3 protein in SI interactions suggests that the nonglycosylated S_3 protein can be used for determining the three-dimensional structure by x-ray crystallography. This strategy circumvents the difficulty often associated with crystallizing glycoproteins, which can be expected in the case of wild-type S proteins because the glycan chain attached to each site of S proteins is heterogeneous (Woodward et al., 1989). Because up to a few micrograms of S_3(N29D) protein per pistil can be produced in transgenic plants and it can be easily purified by a simple two-step procedure (Singh et al., 1991), it would be possible to use the transgenic plants to purify a large quantity of the S_3(N29D) protein for x-ray crystallographic study. Information on the three-dimensional structure of the S_3 protein and other S proteins would be of great value in understanding the structure/function relationship of S proteins and in designing strategies to identify the pollen *S* allele product with which S proteins interact to understand ultimately the molecular detail of SI interactions.

METHODS

Site-Directed Mutagenesis

The site-directed mutagenesis method developed by Ho et al. (1989) was used to change the AAC codon (167 to 169 bp) for Asn-29 of the S_3 protein to a GAC codon for Asp. The previously constructed plasmid pBK-S3 (Huang et al., 1994), which contains an SpeI fragment encompassing −186 to 1052 bp of the S_3 gene of *Petunia inflata* (Coleman and Kao, 1992) ligated into pBluescript II KS+ vector (Stratagene), was used as a template for mutagenesis. Two oligonucleotides were synthesized as primers for polymerase chain reaction (PCR): primer A, 5′-AATACCCAACGACTTTACAAT-3′; primer B, 5′-ATTGTAAA GTCGTTGGGTATT-3′. Except for an A-to-G change in primer A and a T-to-C change in primer B (the underlined positions in the two primer sequences previously shown), primer A and primer B correspond to 157 to 177 bp of the transcribed and nontranscribed strands of the S_3 gene (Coleman and Kao, 1992), respectively. pBK-S3 DNA was amplified in two separate PCRs: one used primer A and the forward pUC/M13 primer of 17 bases obtained from Promega, and the other used primer B and the reverse pUC/M13 primer of 17 bases, which was also obtained from Promega. The reaction conditions were as described by Huang et al. (1994). The two DNA fragments obtained were further amplified together by PCR, using the forward and reverse primers as described by Huang et al. (1994). The DNA fragment obtained was digested with SpeI to release the SpeI fragment of the mutant S_3 gene. The entire coding region contained in the SpeI fragment was sequenced to confirm the AAC-to-GAC change and to ensure

that no other changes had been introduced. The SpeI fragment was then used to replace the corresponding SpeI fragment of the wild-type S_3 gene in pBI-GS3 (Lee et al., 1994) to yield pBI-GS3(N29D).

Plant Material and Transformation

The *P. inflata* plants used in this study were descendants of those described by Ai et al. (1990). The recombinant Ti plasmid pBI-GS3(N29D), which contained the mutant S_3 gene, was electroporated into *Agrobacterium tumefaciens* LBA4404. Infection of leaf discs of *P. inflata* of the S_7S_2 genotype with Agrobacterium and subsequent regeneration through tissue culture were performed as previously described (Huang et al., 1994).

SDS-PAGE

Total protein was extracted from pistils collected from opened flowers in 30 μL of extraction buffer as previously described (Lee et al., 1994). After centrifuging the crude extract at 12,000*g* for 10 min, 25 μL of the supernatant was mixed with an equal volume of 2 × sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue), and the mixture was boiled for 5 min and quenched on ice prior to loading on the gel. An SDS–polyacrylamide slab gel (12% running gel and 4% stacking gel) with a discontinuous buffer system, as described by Laemmli (1970), was used for electrophoresis. After electrophoresis at 70 mA for ~4 hr, the gel was stained in a staining solution (0.125% Coomassie Brilliant Blue R 250, 50% methanol, 10% acetic acid) for 15 hr and destained in a destaining solution (12.5% methanol, 10% acetic acid) for 8 hr. The molecular mass markers were from Bio-Rad.

Cation Exchange Chromatography on a Mono-S Column

Total protein was extracted from freshly collected pistils of opened flowers in 100 μL of extraction buffer according to the method previously described (Lee et al., 1994). The homogenate was mixed with 3 mL of 50 mM sodium phosphate, pH 6.0, and chromatographed on a Mono-S column (HR 5/5) using the fast protein liquid chromatography system from Pharmacia (Piscataway, NJ). Bound proteins were eluted with a linear gradient of 60 mL from 0 to 500 mM NaCl in the same buffer at a flow rate of 1.0 mL/min. Proteins were monitored at $A_{280\,nm}$ with the sensitivity of the detector set to 0.02 absorbance unit full scale. The N-terminal sequence of the Mono-S fraction containing the mutant S_3 protein, S_3(N29D), was determined at The Pennsylvania State University DNA–protein laboratory.

Genomic DNA Isolation and DNA Gel Blot Analysis

Young leaves, weighing 1.2 g, from each plant were used for genomic DNA isolation using a Qiagen cell culture DNA minikit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Ten micrograms of genomic DNA was digested with EcoRI, separated on a 0.8% agarose gel, and transferred to a Biotrans (+) nylon membrane (ICN, Costa Mesa, CA). The conditions for prehybridization, hybridization, and washing of the membrane were identical to those described by Lee et al. (1994). The membrane was exposed on x-ray film at −70°C for 48 hr with an intensifying screen.

Isolation of Total RNA and RNA Gel Blot Analysis

Total RNA isolation and RNA gel blot analysis procedures were performed as previously described by Lee et al. (1994). The membrane was first hybridized with an oligonucleotide probe specific to sense S_3 RNA (Lee et al., 1994). After overnight hybridization, the membrane was washed and exposed on x-ray film at −70°C with an intensifying screen for 48 hr. The amount of radioactivity associated with each hybridizing band was determined using a Betascope (Betagen, Waltham, MA). The bound radiolabeled probe was then removed from the membrane for hybridization with an rDNA probe that encodes 25S rRNA of *P. inflata* (J. Mu and T.-h. Kao, unpublished data). The membrane was exposed on x-ray film at −70°C for 4 hr with an intensifying screen. The amount of radioactivity associated with each hybridizing band was determined using a Betascope. The amount of S_3*(N29D)* RNA in each transgenic plant relative to the amount of S_3 RNA in the S_2S_3 plant was calculated after correction for differences in the total amount of rRNA.

Ribonuclease and Glycan Assays

S proteins were purified by the two-step procedure previously described (Singh et al., 1991). Protein concentrations were determined by the Bradford method (Bradford, 1976) using reagents from Bio-Rad. Ribonuclease activity assays were performed using torula yeast RNA as a substrate as described by Singh et al. (1991). For glycan assay, the purified wild-type S_3 protein and mutant S_3(N29D) protein were electrophoresed in an SDS–polyacrylamide gel as previously described and electroblotted to an Immobilon P membrane (Millipore, Bedford, MA) in an electroblotting buffer (Harlow and Lane, 1988). After the transfer was complete, the blot was fixed in acetic acid–isopropanol–water (10:25:65 [v/v]). To visualize the proteins, the blot was stained with a Coomassie blue staining solution (0.1% Coomassie blue, 1% acetic acid, 40% methanol) for 1 min and destained in a destaining solution (50% methanol) with several changes until the background was clear. After photographing, the blot was completely destained in 100% methanol for the glycan detection assay using the affinoblotting method described by Faye and Chrispeels (1985). Incubation of the blot in a concanavalin A (ConA) solution, washing of the blot, incubation of the blot in a horseradish peroxidase solution, and final washing of the blot were performed as described by Faye and Chrispeels (1985). However, diaminobenzidine (Sigma) was used as a substitute for 4-chloro-naphthol in the staining solution to visualize glycoproteins.

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