

## Ribonuclease Activity of *Petunia inflata* S Proteins Is Essential for Rejection of Self-Pollen

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**S proteins, pistil-specific ribonucleases that cosegregate with S alleles, have previously been shown to control rejection of self-pollen in *Petunia inflata* and *Nicotiana glauca*, two solanaceous species that display gametophytic self-incompatibility. The ribonuclease activity of S proteins was thought to degrade RNA of self-pollen tubes, resulting in the arrest of their growth in the style. However, to date no direct evidence has been obtained. Here, the ribonuclease activity of S<sub>3</sub> protein of *P. inflata* was abolished, and the effect on the pistil's ability to reject S<sub>3</sub> pollen was examined. The S<sub>3</sub> gene was mutagenized by replacing the codon for His-93, which has been implicated in ribonuclease activity, with a codon for asparagine, and the mutant S<sub>3</sub> gene was introduced into *P. inflata* plants of S<sub>1</sub>S<sub>2</sub> genotype. Two transgenic plants produced a level of mutant S<sub>3</sub> protein comparable to that of the S<sub>3</sub> protein produced in self-incompatible S<sub>1</sub>S<sub>3</sub> and S<sub>2</sub>S<sub>3</sub> plants, yet they failed to reject S<sub>3</sub> pollen. The mutant S<sub>3</sub> protein produced in these two transgenic plants did not exhibit any detectable ribonuclease activity. We have previously shown that transgenic plants (S<sub>1</sub>S<sub>2</sub> plants transformed with the wild-type S<sub>3</sub> gene) producing a normal level of wild-type S<sub>3</sub> protein acquired the ability to reject S<sub>3</sub> pollen completely. Thus, the results reported here provide direct evidence that the biochemical mechanism of gametophytic self-incompatibility in *P. inflata* involves the ribonuclease activity of S proteins.**

### INTRODUCTION

Many species of flowering plants possess a prezygotic reproductive barrier, designated gametophytic self-incompatibility, that allows the pistil to recognize and reject self-pollen or pollen from genetically related individuals to prevent self-fertilization (de Nettancourt, 1977). In the Solanaceae family, this self-/non-self-discrimination is controlled by a highly polymorphic S locus. Pollen that bears an S allele identical to one of the two S alleles carried by the pistil suffers growth arrest in the style; only pollen bearing an S allele different from those carried by the pistil grows down the style to the ovary to effect fertilization.

Pistil proteins that cosegregate with S alleles have been identified in a number of solanaceous species (for reviews, see Haring et al., 1990; Singh and Kao, 1992; Sims, 1993). These proteins, named S proteins, exhibit characteristics implicating their involvement in self-incompatibility (Kao and Huang, 1994). Recently, the long-sought direct evidence for the role of S proteins in self-incompatibility has been obtained (Lee et al., 1994; Murfett et al., 1994). These experiments showed that S proteins are necessary and sufficient for the pistil to

reject self-pollen. Specifically, inhibition of S protein production by an antisense S gene in transgenic plants leads to their inability to reject pollen bearing the S allele to which the affected S protein corresponds; the transgenic plants are thus rendered self-compatible (Lee et al., 1994). Conversely, when a gene corresponding to a third and different S allele is introduced into transgenic plants originally containing two S alleles, those plants that express the transgene at a normal level not only reject pollen bearing the endogenous S alleles but also acquire the ability to reject pollen bearing the same S allele as the transgene (Lee et al., 1994; Murfett et al., 1994).

Although the role of S proteins in rejecting self-pollen has now been firmly established, it remains to be determined how S proteins distinguish between self- and non-self-pollen and how this recognition leads to the growth arrest of self-pollen tubes. To address these problems, one must identify the pollen S allele product with which S proteins interact and gain more insight into the structure–function relationship of S proteins. To date, the identity of the pollen S allele remains elusive. However, the unexpected discovery that S proteins are ribonucleases (McClure et al., 1989) has shed much light on the biochemical mechanism of self-rejection. Models have been proposed based on the assumption that self-incompatibility is mediated by the cytotoxic action of the ribonuclease activity

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of S proteins (Haring et al., 1990; Thompson and Kirch, 1992; Sims, 1993; Kao and Huang, 1994). However, the fact that S proteins are ribonucleases does not necessarily mean that the ribonuclease activity is required for their function in self-incompatibility. The activity could be coincidental to the protein. Thus, it is imperative to determine whether or not the ribonuclease activity is essential for the function of S proteins.

This question has been previously addressed using two approaches. First, McClure et al. (1990) compared the fate of radiolabeled pollen RNA in *Nicotiana glauca* pistils after self- or cross-pollination and found that pollen rRNA recovered from self-pollinated pistils was degraded, whereas that from cross-pollinated pistils remained intact. These results are consistent with the hypothesis that the ribonuclease activity of S proteins degrades rRNA of self-pollen tubes, thereby inhibiting their growth in the style. However, the observed degradation of pollen tube rRNA in self-pollinated pistils may equally likely have occurred after the rRNA had been released from the pollen tubes as a result of self-incompatibility interactions (Singh and Kao, 1992; Newbigin et al., 1993). This is because self-pollen tubes frequently burst open after their growth has been arrested, releasing their cytoplasmic contents into the transmitting tract of the style, an area rich in ribonucleases (Singh et al., 1991).

In the second approach, Kowyama et al. (1994) mapped a naturally occurring mutation in a self-compatible variant of *Lycopersicon peruvianum* to the S locus and identified a pistil protein that cosegregated with the mutant  $S_c$  allele. This  $S_c$  protein was found to have no detectable ribonuclease activity. Further, the  $S_c$  protein was found to contain a mutation in which a histidine residue thought to be essential for ribonuclease activity had been changed (cited in Dickinson, 1994). Unfortunately, because the wild-type S allele from which the mutant  $S_c$  allele was derived has not been identified, it was not possible to conclude whether the loss of self-incompatibility was attributable to the negation of the ribonuclease activity or other alterations in the  $S_c$  protein. As a consequence, these results again are consistent with but do not provide direct evidence for the involvement of the ribonuclease activity in self-incompatibility interactions.

One approach to directly ascertain the role of the ribonuclease activity of S proteins in self-incompatibility might be to examine the effect of abolishing the ribonuclease activity of S proteins on their ability to reject self-pollen. Conceivably, one can either produce a mutant S protein by chemical modification or by expressing a mutant S gene in a heterologous system; one could then test the ability of the mutant S protein to inhibit growth of in vitro-germinated pollen tubes. However, because it is difficult to precisely reproduce in an in vitro germination system the exact in vivo milieu that pollen tubes encounter, the inhibitory effect of even wild-type S proteins is not pronounced in in vitro bioassays, and nonspecific inhibition of pollen tube growth is often observed (Jahnen et al., 1989; A. Singh and T.-h. Kao, unpublished results). Thus, the results from this type of bioassay are difficult to interpret.

We previously demonstrated that a *Petunia inflata* plant could acquire new S allele specificity by the expression of a new S gene. This has made possible the use of an in vivo approach to dissect the structure–function relationship of S proteins. Here, we address the question of whether a *P. inflata* plant of  $S_7S_2$  genotype can acquire the ability to reject  $S_3$  pollen if it is transformed with a mutant  $S_3$  gene encoding an  $S_3$  protein lacking ribonuclease activity.

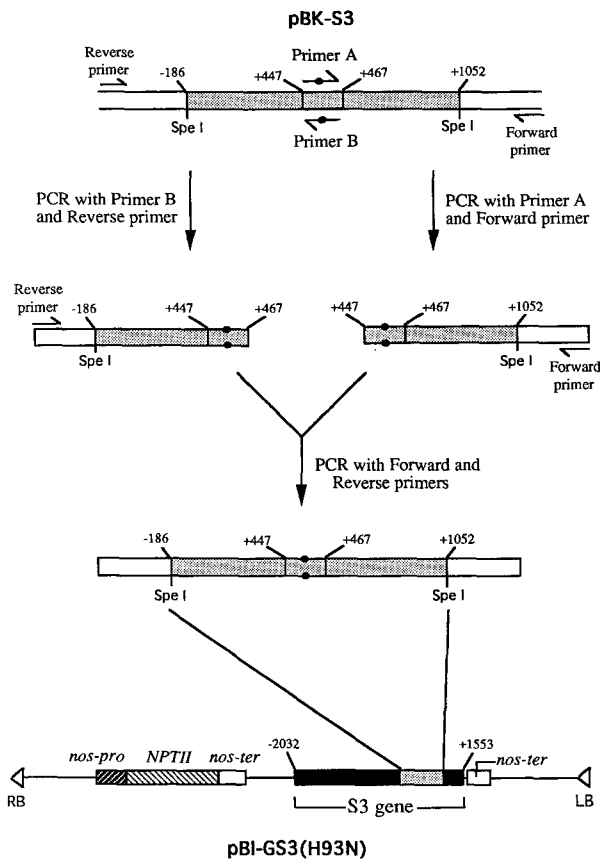
## RESULTS

### Transformation of *P. inflata* Plants of $S_7S_2$ Genotype with a Mutant $S_3$ Gene

His-33 and His-93 of the *P. inflata*  $S_3$  protein (Ai et al., 1990) are the two histidines that are conserved in all the S proteins examined and in two fungal ribonucleases, RNase T2 and RNase Rh, which share sequence similarity with S proteins (Kao, 1993). For the two fungal ribonucleases, these two histidines have been shown to be directly involved in their catalytic function (Kawata et al., 1990; Ohgi et al., 1992). Thus, to abolish the ribonuclease activity of the  $S_3$  protein, we chose to change the CAT codon for His-93 to an AAT codon for asparagine to create a mutant  $S_3$  gene, designated  $S_3(H93N)$  (Figure 1). The Ti plasmid containing the mutant  $S_3$  gene was introduced into *P. inflata* plants of  $S_7S_2$  genotype via *Agrobacterium*-mediated transformation. A total of 162 transgenic plants were chosen for analysis.

### Analysis of Levels of Mutant $S_3$ Protein in Pistils of Transgenic Plants

We previously showed that the expression level of an  $S_3$  transgene in *P. inflata* plants of  $S_7S_2$  genotype was crucial for transgenic plants to acquire  $S_3$  allele specificity (Lee et al., 1994). Only those transgenic plants that produced a level of  $S_3$  protein similar to the level of the  $S_3$  protein produced by nontransgenic  $S_2S_3$  or  $S_7S_3$  plants were able to reject  $S_3$  pollen completely. Therefore, we analyzed pistil proteins of the 162 transgenic plants by cation exchange column chromatography to search for plants that produced the mutant  $S_3$  protein,  $S_3(H93N)$ , at a level similar to that of the  $S_3$  protein produced in  $S_2S_3$  plants. Two such plants, H93N-99 and H93N-132, were found. In addition to producing normal levels of endogenous  $S_1$  and  $S_2$  proteins, these two plants also produced a protein that eluted at a slightly lower salt concentration than the  $S_3$  protein; the elution profile of one of the plants, H93N-99, is shown in Figure 2. The mobility of this protein was found to be identical with that of the  $S_3$  protein when analyzed by SDS-PAGE (results not shown). Further, the first six amino acids at the N terminus of this protein were determined, and the sequence Asn-Phe-Asp-Tyr-Ile-Gln matches perfectly with



**Figure 1.** Construction of Mutant  $S_3$  Gene to Replace the His-93 Codon with an Asparagine Codon.

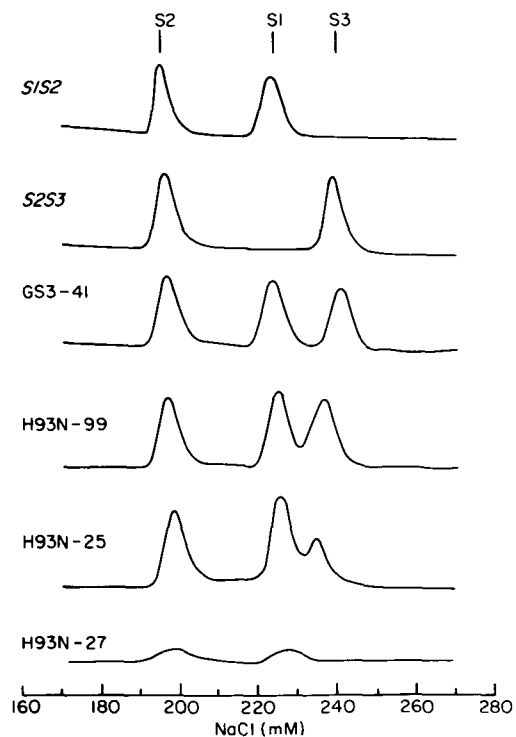
pBK-S3 DNA was amplified by two separate PCR reactions, one using primer A and a forward primer and the other using primer B and a reverse primer, to yield two DNA fragments that overlap in the region spanning 447 to 467 bp of the  $S_3$  gene. The darkened circles indicate the C-to-A change in primer A and the G-to-T change in primer B. The two DNA fragments were further amplified using forward and reverse primers to yield a DNA fragment that contained the SpeI fragment of the  $S_3$  gene, except that the CAT codon for His-93 was replaced by the AAT codon for asparagine. This fragment was used to replace the corresponding fragment in pBI-GS3 (Lee et al., 1994), which is a recombinant Ti plasmid containing -2032 to +1553 bp of the  $S_3$  gene ligated to pBI101 (Clontech), to yield pBI-GS3(H93N). RB, right border; LB, left border; *nos-pro*, promoter of nopaline synthase gene; *nos-ter*, polyadenylation signal of nopaline synthase gene; *NPTII*, coding sequence of neomycin phosphotransferase II gene.

that of the  $S_3$  protein. Thus, we concluded that this protein was the mutant  $S_3$  protein,  $S_3$ (H93N). The shift of the elution position of the  $S_3$ (H93N) protein toward a lower salt concentration relative to that of the  $S_3$  protein is also consistent with the replacement of His-93 of the  $S_3$  protein with a less basic amino acid, Asn-93, in the  $S_3$ (H93N) protein.

Forty-seven transgenic plants were found to produce lower levels of the  $S_3$ (H93N) protein; the elution profile of one of them, H93N-25, is shown in Figure 2. The remaining transgenic plants did not produce any detectable amount of the  $S_3$ (H93N) protein. Three of those plants also produced very low levels of either an endogenous  $S_1$  or  $S_2$  protein, and one plant, H93N-27, produced a very low level of both endogenous  $S_1$  and  $S_2$  proteins (Figure 2). The suppression of the production of endogenous S proteins in these four transgenic plants by the transgene was most likely caused by the cosuppression phenomenon (Napoli et al., 1990).

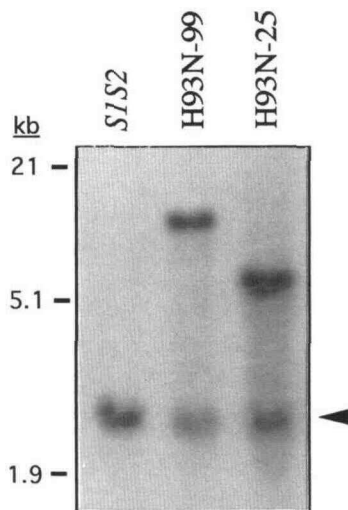
#### DNA Gel Blot Analysis of the Presence of the Transgene and RNA Gel Blot Analysis of Its Transcript Level in Transgenic Plants

To confirm that the transgenic plants indeed carried the transgene and to determine the copy number, a number of



**Figure 2.** Cation Exchange Chromatographic Profiles of S Proteins in Transgenic and Nontransgenic Plants.

Total pistil protein from each plant was chromatographed on a Mono-S column, and the portion of each elution profile containing S proteins is shown.  $S_2S_3$  and  $S_7S_2$  are nontransgenic plants; GS3-41 is a transgenic plant that has previously been shown to have acquired the ability to completely reject  $S_3$  pollen resulting from the expression of the  $S_3$  transgene (Lee et al., 1994); H93N-99, H93N-25, and H93N-27 are representative transgenic plants obtained in this study.



**Figure 3.** Genomic DNA Gel Blot Analysis.

The blot containing EcoRI digests of genomic DNA (10  $\mu$ g per lane) isolated from  $S_1S_2$ , a nontransgenic plant, and H93N-25 and H93N-99, two transgenic plants, was hybridized with  $S_3$  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 conditions used. The DNA length markers are indicated at left in kilobases.

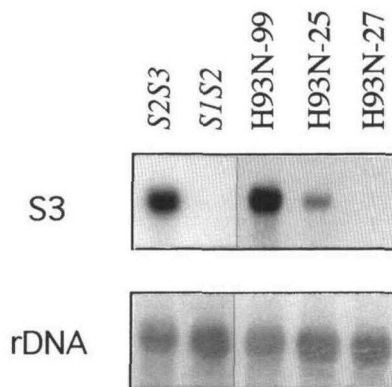
transgenic plants were selected for DNA gel blot analysis. A blot of EcoRI-digested genomic DNA was hybridized with a radiolabeled probe of the full-length  $S_3$  cDNA (Ai et al., 1990). The results for two of the transgenic plants, H93N-99 and H93N-25, and a nontransgenic  $S_1S_2$  plant are shown in Figure 3. Both transgenic plants contained a 2.5-kb hybridizing fragment that was also present in the  $S_1S_2$  plant. This fragment corresponds to the endogenous  $S_2$  gene that cross-hybridized with the  $S_3$  cDNA as a result of sequence similarity. Both transgenic plants contained an additional hybridizing DNA fragment that corresponds to the transgene. Each of these two DNA fragments resulted from one cut within the integrated transgene and a second cut outside the transgene in the genome. Their different sizes indicated different chromosome integration sites of the transgene in the two transgenic plants.

To determine whether the level of the  $S_3$ (H93N) transcript paralleled that of the protein shown in Figure 2, RNA gel blot analysis was performed on total RNA isolated from pistils of a nontransgenic  $S_2S_3$  plant and three transgenic plants, H93N-25, H93N-27, and H93N-99 (Figure 4). H93N-99 contained approximately the same level of  $S_3$  RNA as did the  $S_2S_3$  plant; H93N-25 contained approximately one-fourth the level of  $S_3$  RNA in the  $S_2S_3$  plant; H93N-27, which contained one copy of the transgene, did not contain any detectable level of  $S_3$  RNA. These results are consistent with the results of protein analysis shown in Figure 2.

### Examination of Self-Incompatibility Phenotypes of Transgenic Plants

We first examined whether transgenic plants H93N-99 and H93N-132, which produced a normal level of mutant  $S_3$ (H93N) protein, could reject  $S_3$  pollen. Pollination of these two plants with pollen from  $S_3S_3$  plants produced large fruits, each with  $\sim$ 200 seeds, a number comparable to that obtained from compatible pollination (Table 1). These results suggest that both H93N-99 and H93N-132 did not acquire the ability to reject  $S_3$  pollen. In contrast, GS3-41, a previously obtained transgenic plant (Lee et al., 1994) that produced a similar level of wild-type  $S_3$  protein from the expression of the  $S_3$  transgene (Figure 2), rejected  $S_3$  pollen completely (Table 1). H93N-25 and all the other transgenic plants that produced lesser amounts of  $S_3$ (H93N) protein also produced large fruits, characteristic of compatible pollination, when pollinated with pollen from  $S_3S_3$  plants (Table 1).

All the transgenic plants described above produced normal levels of  $S_1$  and  $S_2$  proteins (Figure 2), and as expected, they remained self-incompatible and rejected pollen from both  $S_1S_1$  and  $S_2S_2$  plants (Table 1). Most of the other 162 transgenic plants analyzed behaved similarly. However, the four transgenic plants mentioned earlier that produced very low levels of either  $S_1$  or  $S_2$  protein or both  $S_1$  and  $S_2$  proteins became self-compatible and failed to reject pollen from  $S_1S_1$  or  $S_2S_2$  or both  $S_1S_1$  and  $S_2S_2$  plants. For example, H93N-27,



**Figure 4.** RNA Gel Blot Analysis of the Expression of the Mutant  $S_3$  Gene.

The blot contains total pistil RNA (10  $\mu$ g per lane) isolated from nontransgenic  $S_2S_3$  and  $S_1S_2$  plants and three transgenic plants, H93N-25, H93N-27, and H93N-99.

(Top) The autoradiogram shows the blot that was hybridized with an oligonucleotide probe specific to sense  $S_3$  RNA (Lee et al., 1994). (Bottom) Results of rDNA probing of the same RNA gel blot. After autoradiography, the bound radiolabeled probe was removed from the blot shown above, and the blot was hybridized with the rDNA probe, which encodes the 25S rRNA of *P. inflata*.

**Table 1.** Results from Cross- and Self-Pollination of Transgenic and Tester Plants of *P. inflata*

Female	Pollen Donor			Self
	$S_1S_1$	$S_2S_2$	$S_3S_3$	
H93N-99	-	-	+	-
H93N-132	-	-	+	-
GS3-41	-	-	-	-
H93N-25	-	-	+	-
H93N-27	+	+	+	+
$S_1S_2$	-	-	+	-
$S_2S_3$	+	-	-	-

- indicates pollination that did not result in fruit set; + indicates pollination that resulted in fruit set.

which produced a very low level of both  $S_1$  and  $S_2$  proteins (Figure 2), failed to reject pollen from both  $S_1S_1$  and  $S_2S_2$  plants (Table 1). These results are consistent with our previous finding using the antisense RNA approach that S proteins are necessary for the pistil to reject self-pollen (Lee et al., 1994). As controls, nontransgenic  $S_1S_2$  and  $S_2S_3$  plants were also pollinated with pollen from  $S_1S_1$ ,  $S_2S_2$ , and  $S_3S_3$  plants, and the expected results were obtained (Table 1).

#### Ribonuclease Activity of Wild-Type S Proteins and Mutant $S_3$ Protein

To investigate whether the  $S_3$ (H93N) protein produced in transgenic plant H93N-99 indeed lacked ribonuclease activity, we purified the  $S_3$ (H93N) protein and examined its ribonuclease activity. As controls, we also examined the ribonuclease activity of purified  $S_1$ ,  $S_2$ , and  $S_3$  proteins. As shown in Table 2, the  $S_3$ (H93N) protein did not show any detectable ribonuclease activity, whereas the  $S_3$ (GS3) protein, which is the  $S_3$  protein expressed from the  $S_3$  transgene in transgenic plant GS3-41 (Figure 2), had ribonuclease activity comparable to that of  $S_3$  protein purified from a nontransgenic  $S_2S_3$  plant. The absence of ribonuclease activity of  $S_3$ (H93N) protein was not caused by inactivation during purification, because the endogenous  $S_1$  and  $S_2$  proteins purified from H93N-99 at the same time had ribonuclease activity comparable to that of  $S_1$ (GS3) and  $S_2$ (GS3) proteins purified from GS3-41 (data not shown). We were also unable to detect ribonuclease activity in another mutant  $S_3$  protein,  $S_3$ (H93R), from a different group of transgenic plants; this protein's His-93 had been replaced by an arginine. (We did not analyze the self-incompatibility phenotypes of this group of transgenic plants because none of them produced a high enough level of  $S_3$ [H93R] protein.) These results are consistent with the previous findings that the histidine of RNase T2 and RNase Rh corresponding to His-93 of the  $S_3$  protein is involved in catalysis (Kawata et al., 1990; Ohgi et al., 1992).

#### DISCUSSION

Although His-93 of the  $S_3$  protein of *P. inflata* had not previously been shown to be essential for ribonuclease activity, sequence comparison between S proteins and two fungal ribonucleases, RNase T2 and RNase Rh, has revealed that this residue corresponds to one of the two histidines of RNase T2 and RNase Rh, which have been shown to be essential for their catalytic function (Kawata et al., 1990; Ohgi et al., 1992). Thus, we targeted this histidine for mutagenesis to abolish the ribonuclease activity of the  $S_3$  protein. We chose asparagine to replace His-93 because of its structural similarity to histidine, thus minimizing the possibility of conformational alteration of the mutant protein. Transformation of  $S_1S_2$  plants with the mutant  $S_3$  gene,  $S_3$ (H93N), resulted in a wide range of expression levels of the transgene in the transgenic plants. The mutant  $S_3$  protein  $S_3$ (H93N) purified from the transgenic plant that expressed the highest level of the transgene, H93N-99, was indeed found not to exhibit any detectable ribonuclease activity. An identical result was obtained with another mutant  $S_3$  protein that had His-93 replaced with arginine. These findings confirm that His-93 of the  $S_3$  protein and most likely its counterpart in other S proteins are essential for catalysis. More importantly, the production of the mutant  $S_3$  protein lacking its ribonuclease activity in transgenic plants allowed us to examine whether the ribonuclease activity of S proteins is required for their function in rejecting self-pollen.

For reasons not known, the level of S proteins is very critical for the pistil's ability to completely reject self-pollen. For example, immature buds that produce S proteins at lower than

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

Protein <sup>a</sup>	Specific Activity <sup>b</sup>
$S_1$ (GS3)	215.3 (4.9)
$S_2$ (GS3)	291.7 (3.9)
$S_3$	261.5 (1.6)
$S_3$ (GS3)	227.1 (3.9)
$S_3$ (H93N)	0
$S_3$ (H93R)	0

<sup>a</sup> S proteins were purified from the following sources:  $S_1$ (GS3),  $S_2$ (GS3), and  $S_3$ (GS3) are from a previously obtained transgenic plant, GS3-41, which expressed an  $S_3$  transgene in addition to the endogenous  $S_1$  and  $S_2$  genes (Lee et al., 1994);  $S_3$  is from an  $S_2S_3$  plant;  $S_3$ (H93N) is a mutant  $S_3$  protein that has His-93 replaced by an Asn from transgenic plant H93N-99;  $S_3$ (H93R) is a mutant  $S_3$  protein that has His-93 replaced by an Arg from a transgenic plant H93R-112.

<sup>b</sup> Specific activity is expressed as the  $A_{260}$  units of torula yeast RNA released into acid soluble form by 1.0 mg of the protein in 1 min at 37°C in 10 mM sodium phosphate, pH 7.0. Each value is the mean of three replicates. The standard error of the mean is given within parentheses.

the normal level produced in mature flowers cannot reject self-pollen completely (Clark et al., 1990). We previously found that  $S_1S_2$  plants transformed with the  $S_3$  gene must also produce the  $S_3$  protein to a level comparable to that produced in mature flowers of  $S_1S_3$  or  $S_2S_3$  plants to acquire the ability to completely reject  $S_3$  pollen (Lee et al., 1994). Thus, it is important to use the transgenic plants that produce a normal level of the mutant  $S_3$  protein to ascertain their ability to reject  $S_3$  pollen, so that we can rule out the possibility that their failure to reject  $S_3$  pollen was a result of an insufficient amount of the mutant  $S_3$  protein produced. Although only two such transgenic plants, H93N-99 and H93N-132, were found, they proved to be informative.

The amount of  $S_3$ (H93N) protein produced in these two transgenic plants was comparable to that of  $S_3$  protein produced in nontransgenic  $S_1S_3$  and  $S_2S_3$  plants, as well as in a previously obtained transgenic plant GS3-41. However, the former could not reject  $S_3$  pollen, whereas the latter completely rejected  $S_3$  pollen. Because the mutant  $S_3$  protein produced in H93N-99 and H93N-132 differs from the wild-type  $S_3$  protein produced in GS3-41,  $S_1S_3$ , and  $S_2S_3$  plants in its lack of ribonuclease activity (resulting from the replacement of His-93 with Asn-93), the results reported here strongly suggest that the ribonuclease activity of S proteins is essential for their function in rejecting self-pollen. The results thus provide direct evidence that the biochemical mechanism of gametophytic self-incompatibility in solanaceous species involves the ribonuclease activity of S proteins.

Our finding of an essential role of the ribonuclease activity of S proteins in rejecting self-pollen provides an answer to one of the two key questions in self-incompatibility mentioned earlier regarding the biochemical mechanism of self-rejection; however, it does not provide an answer to the other key question regarding how S proteins distinguish self-pollen from non-self-pollen. A currently favored model proposes that the S allele-specific rejection of self-pollen lies in the specific uptake of S proteins into the cytoplasm of pollen tubes; the product of the pollen S allele, serving as a receptor for S proteins, only allows S proteins bearing the same allele to enter the cytoplasm to degrade RNA (Dickinson, 1994). The validity of this model can be confirmed only after the pollen S allele product has been identified.

Our finding reported here also raises the issue regarding which feature(s) of S proteins distinguishes them from other plant ribonucleases with which they share sequence similarity. Sequence comparison and phylogenetic analysis of the S-like ribonucleases and S proteins have revealed that most of the S-like ribonucleases are more similar to each other than to S proteins and that these two classes of ribonucleases fall into two distinct lineages (Taylor et al., 1993; Green, 1994). These results are consistent with the distinct function of S proteins from the S-like ribonucleases (although the function of none of the S-like ribonucleases has been determined, they are unlikely to be involved in self-incompatibility). However, one of the S-like ribonucleases, RNase X2 of *P. inflata*, exhibits a very high degree of sequence similarity with S proteins and

is also a pistil-specific extracellular ribonuclease (Lee et al., 1992). In fact, two *N. alata* S proteins are more similar to RNase X2 than they are to the other S proteins (Kao, 1993).

The issue raised above is thus especially pertinent to RNase X2 and perhaps to other yet to be identified S-like ribonucleases with similar properties to RNase X2. One feature that clearly distinguishes the S gene from the RNase X2 gene and the genes encoding other S-like ribonucleases is the high degree of polymorphism displayed by the former and the monomorphism displayed by the latter. This suggests that S proteins are endowed with the recognition domain for the pollen S allele product, and if the model described above is correct, they can enter the pollen tubes carrying a matching S allele to exert their cytotoxic action, whereas S-like ribonucleases do not contain the pollen recognition domain and cannot exert cytotoxicity on pollen tubes even if they are also present in the intercellular space of the transmitting tissue. In the future, the *in vivo* approach we used in our study can also be used to examine S allele specificities of chimeric S genes in transgenic plants. This will allow researchers to identify the pollen recognition domain or the S allele specificity domain of S proteins. This information may aid the design of experiments to identify the pollen S allele product.

Although ribonucleases were favored subjects for much of the seminal work on protein folding and tertiary structure, interest in them waned in the 1970s because their biological significance *in situ* was not understood. However, there is growing recognition of the possible involvement of extracellular ribonucleases in the regulation of growth and development in plants (Farkas, 1982) and animals (Benner and Allemann, 1989). In this context, our demonstration that a self-incompatible solanaceous species employs ribonucleases (S proteins) to reject self-pollen to prevent inbreeding has provided direct evidence for the involvement of ribonucleases in regulation of growth and development in plants.

## METHODS

### Site-Directed Mutagenesis

An *SpeI* fragment encompassing –186 to 1052 bp of the  $S_3$  gene of *Petunia inflata* (Coleman and Kao, 1992) was ligated into pBluescript II KS+ vector (Stratagene) to yield pBK-S3. The strategy for site-directed mutagenesis to change the CAT codon (457 to 459 bp) for the invariant histidine, His-93, of the  $S_3$  protein to an AAT codon for asparagine followed that developed by Ho et al. (1989). Two oligonucleotides with the following sequences were synthesized to use as primers for polymerase chain reaction (PCR): primer A, 5'-ATACAATAAAAATGGAA-TATG-3'; primer B, 5'-CATATCCATTTTATTGTAT-3'. Primer A and primer B correspond to 447 to 467 bp of the nontranscribed and transcribed strands of the  $S_3$  gene (Coleman and Kao, 1992), respectively, except for a C-to-A change in primer A and a G-to-T change in primer B (the underlined positions in the two primer sequences shown above). The other two primers used were forward and reverse universal primers of 17 bases obtained from Promega. pBK-S3 DNA was amplified in

two separate PCRs (40 cycles each), one with primer A and the forward primer and the other with primer B and the reverse primer.

The reactions were conducted in a 100- $\mu$ L solution containing 0.1  $\mu$ g of DNA, 400 pM each of the primers, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, and 1.5 mM MgCl<sub>2</sub>. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 44°C for 1 min, and extension at 72°C for 2 min, except that denaturation was for 2 min in the first cycle. Immediately following the last cycle, the samples were left at 72°C for 5 min to allow the reactions to be completed. The two DNA fragments obtained were purified by low-melting agarose gel electrophoresis, and 0.3  $\mu$ g of each DNA fragment was amplified by PCR together using 400 pM of forward and reverse primers under the same conditions described above. The DNA fragment obtained was digested with SpeI to release the SpeI fragment of the mutant S<sub>3</sub> gene. The entire coding region contained in the SpeI fragment was sequenced to confirm the CAT-to-AAT mutation 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<sub>3</sub> gene in pBI-GS3 (Lee et al., 1994) to yield pBI-GS3(H93N).

#### Plant Material and Transformation

The S genotypes of the *P. inflata* plants used in this study were determined by pollination with tester plants of known genotypes (Ai et al., 1990) and by PCR analysis of genomic DNA using allele-specific primers. The recombinant Ti plasmid pBI-GS3(H93N), which contained the mutant S<sub>3</sub> gene, was electroporated into *Agrobacterium tumefaciens* LBA4404 as previously described by S. Singh, T.-h. Kao, and J.-J. Lin in a 1993 issue of *Focus* published by Life Technologies, Inc. (Gaithersburg, MD). Leaf discs of *P. inflata* with the S<sub>1</sub>S<sub>2</sub> genotype were infected with the *Agrobacterium* by the cocultivation method (Horsch et al., 1985) on Murashige and Skoog (MS) medium (Sigma) supplemented with benzylaminopurine (1.0 mg/L) and naphthalene acetic acid (75  $\mu$ g/L). Shoots were regenerated on fresh MS medium supplemented with kanamycin (100  $\mu$ g/mL) and carbenicillin (500  $\mu$ g/mL). Regenerated shoots were transferred to hormone-free MS medium containing the same concentrations of antibiotics to induce root formation.

#### Purification of S Proteins

Total pistil protein was extracted from 30 pistils of each plant in 1 mL of extraction buffer as previously described (Lee et al., 1994), and the homogenate was applied to a column (1.6  $\times$  10 cm) of Biogel P-60 (Bio-Rad) that had been equilibrated with 50 mM sodium phosphate, pH 6.0. Fractions containing S proteins, as determined by SDS-PAGE, were collected and chromatographed on a Mono-S column (HR 5/5) using the fast protein liquid chromatography system from Pharmacia. The bound proteins were eluted with a linear gradient of 0 to 500 mM NaCl in the same buffer at a flow rate of 1.0 mL/min. Proteins were monitored at A<sub>280 nm</sub> with the sensitivity of the detector set to 0.1 absorbance-unit-full-scale. The N-terminal sequence of the Mono-S fraction containing the mutant S<sub>3</sub> protein, S<sub>3</sub>(H93N), was determined at the Macromolecular Core Facility of the Pennsylvania State University Hershey Medical Center.

#### Isolation of Genomic DNA and DNA Blot Analysis

Genomic DNA was isolated from 5 g of young leaves freshly collected from each plant as described by Lee et al. (1994). Genomic DNA (10

$\mu$ g) was digested with EcoRI, separated on a 0.8% agarose gel, and transferred to a Biotrans (+) nylon membrane (ICN, Costa Mesa, CA). Prehybridization, hybridization, and washing of the membrane were conducted as previously described (Lee et al., 1994). The membrane was exposed on x-ray film at -70°C for 72 hr with an intensifying screen.

#### Isolation of Total RNA from Pistils and RNA Gel Blot Analysis

The procedures for isolation of total RNA and for RNA blot analysis were identical to those previously described (Ai et al., 1990; Lee et al., 1994). The membrane was first hybridized with an oligonucleotide probe specific to sense S<sub>3</sub> RNA (Lee et al., 1994). After washing, the membrane was exposed on x-ray film at -70°C with an intensifying screen for 16 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, and the membrane was rehybridized with the ribosomal DNA probe that encodes 25S rRNA of *P. inflata* (J. Mu and T.-h. Kao, unpublished results). The membrane was exposed on x-ray film at -70°C for 30 min with an intensifying screen. The amount of radioactivity associated with each hybridizing band was determined using a Betascope. The amount of S<sub>3</sub> RNA in each transgenic plant relative to the amount of S<sub>3</sub> RNA in the S<sub>2</sub>S<sub>3</sub> plant was calculated after correction for differences in the total amount of rRNA.

#### Ribonuclease Assay

All S proteins were purified by the two-step procedure described above. For S<sub>3</sub>(H93N) protein, the Mono-S fractions containing it were rechromatographed on the same column to remove contaminating S<sub>1</sub> protein. 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 substrate as previously described (Singh et al., 1991).

#### ACKNOWLEDGMENTS

We thank David Tu for use of his fast protein liquid chromatography system, and Andrew Clark, Daniel Cosgrove, Richard Cyr, Simon Gilroy, Andrew McCubbin, and Steve Schaeffer for valuable comments on the manuscript. H.-S.L. was partially supported by the National Science Foundation (NSF) Postdoctoral Fellowship in Plant Biology Grant No. BIR-9303646. B.K. was supported by a Third Country Graduate Fellowship from Pioneer Hi-Bred International, Inc. The research was supported by a grant from NSF (No. IBN-9220145) to T.-h.K.

Received March 22, 1994; accepted May 4, 1994.

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