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 alata, 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 S3 protein of Petunia inflata was abolished, and the effect on the pistil's ability to reject S3 pollen was examined. The S3 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 S3 gene was introduced into Petunia inflata plants of S3S3 genotype. Two transgenic plants produced a level of mutant S3 protein comparable to that of the S3 protein produced in self-incompatible S2S2 and S2S3 plants, yet they failed to reject S3 pollen. The mutant S3 protein produced in these two transgenic plants did not exhibit any detectable ribonuclease activity. We have previously shown that transgenic plants (S1S2 plants transformed with the wild-type S3 gene) producing a normal level of wild-type S3 protein acquired the ability to reject S3 pollen completely. Thus, the results reported here provide direct evidence that the biochemical mechanism of gametophytic self-incompatibility in Petunia 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-innon-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...
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 alata 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 clease activity had been changed (cited in Dickinson, 1994). Unfortunately, because the wild-type S allele from which the mutant S 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 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 S3S3 genotype can acquire the ability to reject S3 pollen if it is transformed with a mutant S3 gene encoding an S3 protein lacking ribonuclease activity.

RESULTS

Transformation of P. inflata Plants of S2S2 Genotype with a Mutant S3 Gene

His-33 and His-93 of the P. inflata S3 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., 1989; Ohgi et al., 1992). Thus, to abolish the ribonuclease activity of the S3 protein, we chose to change the CAT codon for His-93 to an AAT codon for asparagine to create a mutant S3 gene, designated S3(H93N) (Figure 1). The Ti plasmid containing the mutant S3 gene was introduced into P. inflata plants of S2S2 genotype via Agrobacterium-mediated transformation. A total of 162 transgenic plants were chosen for analysis.

Analysis of Levels of Mutant S3 Protein in Pistils of Transgenic Plants

We previously showed that the expression level of an S2 transgene in P. inflata plants of S1S2 genotype was crucial for transgenic plants to acquire S3 allele specificity (Lee et al., 1994). Only those transgenic plants that produced a level of S3 protein similar to the level of the S3 protein produced by nontransgenic S2S2 or S1S2 plants were able to reject S3 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 S3 protein, S3(H93N), at a level similar to that of the S3 protein produced in S2S2 plants. Two such plants, H93N-99 and H93N-132, were found. In addition to producing normal levels of endogenous S1 and S2 proteins, these two plants also produce a protein that eluted at a slightly lower salt concentration than the S3 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 S3 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 S3 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 S3 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 S3 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 S3 gene ligated to pBI101 (Clonetech), 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 S3 protein. Thus, we concluded that this protein was the mutant S3 protein, S3(H93N). The shift of the elution position of the S3(H93N) protein toward a lower salt concentration relative to that of the S3 protein is also consistent with the replacement of His-93 of the S3 protein with a less basic amino acid, Asn-93, in the S3(H93N) protein.

Forty-seven transgenic plants were found to produce lower levels of the S3(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 S3(H93N) protein. Three of those plants also produced very low levels of either an endogenous S1 or S2 protein, and one plant, H93N-27, produced a very low level of both endogenous S1 and S2 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. S3-S2 and S1-S2 are nontransgenic plants; GS3-41 is a transgenic plant that has previously been shown to have acquired the ability to completely reject S3 pollen resulting from the expression of the S3 transgene (Lee et al., 1994); H93N-99, H93N-25, and H93N-27 are representative transgenic plants obtained in this study.
transgenic plants were selected for DNA gel blotted analysis. A blot of EcoRI-digested genomic DNA was hybridized with a radiolabeled probe of the full-length S3 cDNA (Ai et al., 1990). The results for two of the transgenic plants, H93N-99 and H93N-25, and a nontransgenic S,S2 plant are shown in Figure 3. Both transgenic plants contained a 2.5-kb hybridizing fragment that was also present in the S,S2 plant. This fragment corresponds to the endogenous S2 gene that cross-hybridized with the S3 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 S3(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 S2S3 plant and three transgenic plants, H93N-25, H93N-27, and H93N-99 (Figure 4). H93N-99 contained approximately the same level of S3 RNA as did the S2S3 plant; H93N-25 contained approximately one-fourth the level of S3 RNA in the S2S3 plant; H93N-27, which contained one copy of the transgene, did not contain any detectable level of S3 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 S3(H93N) protein, could reject S3 pollen. Pollination of these two plants with pollen from S2S3 plants produced large fruits, each with ~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 S3 pollen. In contrast, GS3-41, a previously obtained transgenic plant (Lee et al., 1994) that produced a similar level of wild-type S3 protein from the expression of the S3 transgene (Figure 2), rejected S3 pollen completely (Table 1). H93N-25 and all the other transgenic plants that produced lesser amounts of S3(H93N) protein also produced large fruits, characteristic of compatible pollination, when pollinated with pollen from S2S3 plants (Table 1).

All the transgenic plants described above produced normal levels of S1 and S2 proteins (Figure 2), and as expected, they remained self-incompatible and rejected pollen from both S1S1 and S2S2 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 S1 or S2 protein or both S1 and S2 proteins became self-compatible and failed to reject pollen from S1S1 or S2S2 or both S1S1 and S2S2 plants. For example, H93N-27,
Table 1. Results from Cross- and Self-Pollination of Transgenic and Tester Plants of *P. inflata*

<table>
<thead>
<tr>
<th>Female</th>
<th>Pollen Donor</th>
<th>S1 S1</th>
<th>S2 S2</th>
<th>S3 S3</th>
<th>Self</th>
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<tbody>
<tr>
<td>H93N-99</td>
<td>- - - -</td>
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<tr>
<td>H93N-132</td>
<td>- - - -</td>
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<tr>
<td>GS3-41</td>
<td>- - - -</td>
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<tr>
<td>H93N-25</td>
<td>- - - -</td>
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<tr>
<td>H93N-27</td>
<td>+ + + +</td>
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<td>S1 S2</td>
<td>- - - -</td>
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<tr>
<td>S2 S2</td>
<td>- - - -</td>
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- indicates pollination that did not result in fruit set; + indicates pollination that resulted in fruit set.

which produced a very low level of both S1 and S2 proteins (Figure 2), failed to reject pollen from both S1S1 and S2S2 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 S1S1 and S2S2 plants were also pollinated with pollen from S1S1, S2S2, and S2S2 plants, and the expected results were obtained (Table 1).

Ribonuclease Activity of Wild-Type S Proteins and Mutant S3 Protein

To investigate whether the S3(H93N) protein produced in transgenic plant H93N-99 indeed lacked ribonuclease activity, we purified the S3(H93N) protein and examined its ribonuclease activity. As controls, we also examined the ribonuclease activity of purified S1, S2, and S3 proteins. As shown in Table 2, the S3(H93N) protein did not show any detectable ribonuclease activity, whereas the S3(GS3) protein, which is the S3 protein expressed from the S3 transgene in transgenic plant GS3-41 (Figure 2), had ribonuclease activity comparable to that of S3 protein purified from a nontransgenic S2S2 plant. The absence of ribonuclease activity of S3(H93N) protein was not caused by inactivation during purification, because the endogenous S1 and S2 proteins purified from H93N-99 at the same time had ribonuclease activity comparable to that of S1(GS3) and S2(GS3) proteins purified from GS3-41 (data not shown). We were also unable to detect ribonuclease activity in another mutant S3 protein, S3(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 S3[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 S3 protein is involved in catalysis (Kawata et al., 1990; Ohgi et al., 1992).

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

<table>
<thead>
<tr>
<th>Protein a</th>
<th>Specific Activity b</th>
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<tbody>
<tr>
<td>S1(GS3)</td>
<td>215.3 (4.9)</td>
</tr>
<tr>
<td>S2(GS3)</td>
<td>291.7 (3.9)</td>
</tr>
<tr>
<td>S3</td>
<td>261.5 (1.6)</td>
</tr>
<tr>
<td>S3(GS3)</td>
<td>227.1 (3.9)</td>
</tr>
<tr>
<td>S3(H93N)</td>
<td>0</td>
</tr>
<tr>
<td>S3(H93R)</td>
<td>0</td>
</tr>
</tbody>
</table>

* a S proteins were purified from the following sources: S1(GS3), S2(GS3), and S3(GS3) are from a previously obtained transgenic plant, GS3-41, which expressed an S3 transgene in addition to the endogenous S and S2 genes (Lee et al., 1994); S3 is from an S2S2 plant; S3(H93N) is a mutant S3 protein that has His-93 replaced by an Asn from transgenic plant H93N-99; S3(H93R) is a mutant S3 protein that has His-93 replaced by an Arg from a transgenic plant H93R-112.

b Specific activity is expressed as the A260 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.

**DISCUSSION**

Although His-93 of the S3 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 S3 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 S1S2 plants with the mutant S3 gene, S3(H93N), resulted in a wide range of expression levels of the transgene in the transgenic plants. The mutant S3 protein S3(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 S3 protein that had His-93 replaced with arginine. These findings confirm that His-93 of the S3 protein and most likely its counterpart in other S proteins are essential for catalysis. More importantly, the production of the mutant S3 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
the normal level produced in mature flowers cannot reject self-
pollen completely (Clark et al., 1990). We previously found that
S1S2 plants transformed with the S2 gene must also produce the S3 protein to a level comparable to that produced in ma-
ture flowers of S1S3 or S2S2 plants to acquire the ability to
completely reject S3 pollen (Lee et al., 1994). Thus, it is im-
portant to use the transgenic plants that produce a normal level
of the mutant S3 protein to ascertain their ability to reject S3
pollen, so that we can rule out the possibility that their failure
to reject S3 pollen was a result of an insufficient amount of
the mutant S3 protein produced. Although only two such
transgenic plants, H93N-99 and H93N-132, were found, they
proved to be informative.

The amount of S3(H93N) protein produced in these two
transgenic plants was comparable to that of S3 protein pro-
duced in nontransgenic S2S2 and S2S3 plants, as well as in
a previously obtained transgenic plant GS3-41. However, the
former could not reject S3 pollen, whereas the latter com-
pletely rejected S3 pollen. Because the mutant S3 protein
produced in H93N-99 and H93N-132 differs from the wild-type
S3 protein produced in GS3-41, S2S2, and S2S3 plants in its
lack of ribonuclease activity (resulting from the replacement
of His-93 with Asn-93), the results reported here strongly sug-
gest that the ribonuclease activity of S proteins is essential
for their function in rejecting self-pollen. The results thus pro-
vide direct evidence that the biochemical mechanism of
gametophytic self-incompatibility in solanaceous species in-
volves 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; how-
ever, 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 similar-
ity. 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 pro-
teins 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 Petunia inflata, exhib-
ts 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 monomor-
phism displayed by the latter. This suggests that S proteins
are endowed with the recognition domain for the pollen S al-
lele product, and if the model described above is correct, they
can enter the pollen tubes carrying a matching S allele to ex-
ert their cytotoxic action, whereas S-like ribonucleases do not
contain the pollen recognition domain and cannot exert cyto-
toxicity 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 recog-
nition 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, in-
terest in them waned in the 1970s because their biological
significance in situ was not understood. However, there is grow-
ing recognition of the possible involvement of extracellular
ribonucleases in the regulation of growth and development
in plants (Farkas, 1982) and animals (Benner and Alleman,
1989). In this context, our demonstration that a self-incom-
patible 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 S2 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 S2 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 poly-
merase chain reaction (PCR): primer A, 5'-ATACAATAAATGGAA-
TATG-3'; primer B, 5'-CATATCCATCTTTATTGAT-3'. Primer A and
primer B correspond to 447 to 467 bp of the nontranscribed and tran-
scribed strands of the S3 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 for-
ward primer and the other with primer B and the reverse primer.

The reactions were conducted in a 100-μL solution containing 0.1
μg of DNA, 400 pM each of the primers, 50 mM KCl, 10 mM Tris-HCl,
ph 8.0, 0.1% Triton X-100, and 1.5 mM MgCl2. 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 elec-
trophoresis, and 0.3 μg 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 Spel to release the Spel fragment of the mutant S3 gene. The
entire coding region contained in the Spel fragment was sequenced
to confirm the CAT-teAAT mutation and to ensure that no other changes
had been introduced. The Spel fragment was then used to replace
the corresponding Spel fragment of the wild-type S3 gene in pB1-GS3
(Lee et al., 1994) to yield pB1-GS3(H93N).

Plant Material and Transformation

The S genotypes of the P. inflata plants used in this study were deter-
mined 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 pB1-GS3(H93N), which contained
the mutant S3 gene, was electroporated into Agrobacterium tumefa-
ciens LBA4404 as previously described by S. Singh., T-h. Kao, and
(Gaithersburg, MD). Leaf discs of P. inflata with the S3/S2 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 μg/L). Shoots were regenerated on fresh MS medium
supplemented with kanamycin (100 μg/mL) and carbenicillin (500
μg/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 x 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 A280 nm, 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 S3 protein, S3(H93N), was determined
at the Macromolecular Core Facility of the Pennsylvania State Univer-
sity 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
μg) 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 S3 RNA (Lee et al., 1994). After washing, the
membrane was exposed on x-ray film at -70°C with an intensifying
screen for 18 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 radioac-
tivity associated with each hybridizing band was determined using an
intensifying screen. The amount of S2 RNA in each transgenic plant rela-
tive to the amount of S3 RNA in the S3S2 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 S3(H93N), the Mono-S fractions containing it were rechrom-
atographed on the same column to remove contaminating S1 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 previ-
ously described (Singh et al., 1991).

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