The Plant Cell, Vol. 10, 1723–1731, October 1998, www.plantcell.org

Identification of Residues in a Hydrophilic Loop of the *Papaver rhoeas* S Protein That Play a Crucial Role in Recognition of Incompatible Pollen

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The self-incompatibility response involves *S* allele–specific recognition between stigmatic S proteins and incompatible pollen. This response results in pollen inhibition. Defining the amino acid residues within the stigmatic S proteins that participate in *S* allele–specific inhibition of incompatible pollen is essential for the elucidation of the molecular basis of the self-incompatibility response. We have constructed mutant derivatives of the S_1 protein from *Papaver rhoeas* by using site-directed mutagenesis and have tested their biological activity. This has enabled us to identify amino acid residues in the stigmatic S proteins of *P. rhoeas* that are required for S-specific inhibition of incompatible pollen. We report here the identification of several amino acid residues in the predicted hydrophilic loop 6 of the *P. rhoeas* stigmatic S_1 protein that are involved in the inhibition of S_1 pollen. Mutation of the only hypervariable amino acid, which is situated in this loop, resulted in the complete loss of ability of the S protein to inhibit S_1 pollen. This clearly demonstrates that this residue plays a crucial role in pollen recognition and may also participate in defining allelic specificity. We have also established the importance of highly conserved amino acids adjacent to this hypervariable site. Our studies demonstrate that both variable and conserved amino acids in the region of the S protein corresponding to surface loop 6 are key elements that play a role in the recognition and inhibition of incompatible pollen in the pollen–pistil self-incompatibility reaction.

INTRODUCTION

Self-incompatibility (SI) in the field poppy (*Papaver rhoeas*) is determined gametophytically by a multiallelic *S* locus (Lawrence et al., 1978). It is proposed that genes residing at the *S* locus encode a stigmatic S protein and a corresponding pollen S protein. Both proteins are responsible for a highly specific recognition event leading to rejection of self-pollen (Franklin et al., 1995). Previous studies of wild populations suggest that there are ~40 to 50 different *S* alleles present in each population, and comparative studies suggest that the overall figure for the species is ~65 (Lane and Lawrence, 1993).

We have reported the cloning of cDNAs for the S_1, S_3, S_7 (partial sequence), and S_8 alleles of the stigmatic *S* gene, together with Sn_1, a putative stigmatic *S* gene from *P. nudicaule* (Foote et al., 1994; Walker et al., 1996; Kurup et al., 1998). Several of these genes have been expressed in *Escherichia coli* to produce recombinant S proteins. These have been isolated and demonstrated to be biologically active because they specifically inhibit pollen of the same *S* genotype in an in vitro bioassay. The S proteins are small (~15 kD) secreted proteins; some are modified by N-glycosylation. Comparison of the amino acid sequences derived from the cloned *S* gene cDNAs has revealed that they are highly polymorphic, sharing between 50 and 65% identity. Nevertheless, they are predicted to adopt virtually indistinguishable secondary structures (Walker et al., 1996; Kurup et al., 1998). These are comprised of a series of six β strands with two α-helical regions located at the C terminus and linked by seven hydrophilic loops, which exhibit high surface probability.

The product of the pollen *S* gene interacts with the stigmatic S protein; however, this product has yet to be identified, although it is believed to be a receptor of some type. We have recently identified a pollen plasma membrane proteoglycan that interacts with the S protein, although not in an *S* allele–specific manner. This S protein binding protein is thought to be an accessory receptor with a role in mediating the specific interaction between the stigma and pollen S

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proteins (Hearn et al., 1996). Studies have demonstrated that this recognition event activates a calcium-based signaling pathway (Franklin-Tong et al., 1993, 1995, 1997) and is associated with phosphorylation of a number of pollen proteins. These events lead to the rapid arrest of pollen tube growth typical of an incompatible pollination (Rudd et al., 1996, 1997).

Currently, an area of crucial importance in the study of SI is defining the molecular basis of *S* allele specificity together with identifying the pollen *S* gene product. Recently, a number of groups have addressed these areas of concern in the SI RNases (S RNases) of the Solanaceae. Although the control of the SI system in the Solanaceae is genetically identical to that of *P. rhoeas*, mechanistically the systems are entirely different. There is no detectable homology between the S RNases and the poppy S proteins. Sequence alignments across a range of S RNases have revealed that these proteins possess five highly conserved domains and two hypervariable domains. A number of studies have investigated the role of the hypervariable regions in the control of allele-specific recognition of pollen. In an elegant experiment, Matton et al. (1997) substituted the hypervariable domains of *Solanum chacoense* S_{11} with those of S_{13}. The result of this substitution was that the transgenic recombinant allele behaved as a fully functional S_{13} allele. This led these authors to conclude that allelic specificity can be determined by the hypervariable regions alone. Although their observations are consistent with this conclusion, as Verica et al. (1998) point out, this result may have been influenced by the particular combination of *S* alleles used in the study.

In comparison with all *S* alleles that have been characterized to date, S_{11} and S_{13} of *S. chacoense* are remarkably similar because they differ in only 10 of 190 amino acids. When similar domain swap experiments were conducted between more divergent pairs of alleles, they did not acquire the new specificity, even though the hybrid proteins retained ribonuclease activity and lost their specificity (Kao and McCubbin, 1996; Zurek et al., 1997). Such observations indicate that swap experiments between more divergent pairs of alleles can result in disruption of recognition. This implies that allelic recognition may not be localized to any particular domain of the S RNase and has led to the suggestion that residues outside of the two designated hypervariable domains may also participate in S specificity (Verica et al., 1998).

To elucidate the molecular basis of the pollen–pistil interaction in poppy, which is the next stage, we are attempting to define the amino acid residues within the stigmatic S proteins that are essential for the recognition of incompatible pollen. We have constructed mutant derivatives of the S proteins and tested their biological activity. In this study, we demonstrate that certain amino acids are crucial for S-specific activity, because some of these mutant S proteins are defective in their inhibition of incompatible pollen. Our studies provide evidence that both variable and conserved amino acids are important in the pollen–pistil SI interaction.

RESULTS

Choice of Amino Acid Residues for Mutation in the S_1 Protein

Our studies to date indicate that variations in amino acid sequence are likely to be responsible for the different specificities of the alleles of the *P. rhoeas* S protein (Foote et al., 1994; Franklin-Tong et al., 1995; Walker et al., 1996). Therefore, we compared the available poppy S protein sequences to identify variable residues that might participate in *S* allele–specific recognition of incompatible pollen. These sequence alignments are shown in Figure 1. They reveal a number of blocks of complete sequence identity or similarity interspersed with more variable regions. Figure 1 also illustrates the secondary structure predictions for the S proteins based on these sequences. We were particularly interested in any variable amino acids present in the predicted hydrophilic surface loops, because these may be readily available for interaction with the pollen *S* gene product. It is evident from Figure 1 that despite the overall high level of sequence polymorphism between the *S* alleles, only one amino acid residue, corresponding to Asp at position 79 in loop 6 of S_1, is completely variable across these five sequences (indicated by gray shading). The sequence alignment also indicates that the S_3 protein possesses an additional amino acid in loop 5. Although loop 5 is not completely polymorphic across all five alleles, it does exhibit a significant degree of variability.

On the basis of these observations, a program of mutagenesis, which is described below, was devised. We have indicated the positions of mutations made to the S_1 protein described here in gray blocks in Figure 1. We were aware of potential problems with activity if the mutations affected folding of the protein, and so all substitutions were, as far as possible, conservative, or they involved replacing the amino acid residues present in the S_1 sequence with those found at the corresponding position in the S_3 or S_8 sequences. Furthermore, because the majority of the mutations were targeted to the predicted hydrophilic surface loops, they were less likely to adversely interfere with the folding of the recombinant proteins. The prime candidate for mutation in the S_1 product was Asp-79 in loop 6. This is because it is the only hypervariable residue identified by the *S* allele sequence alignment; in addition, it was predicted to be in a surface loop. Subsequent mutations were incorporated in loop 5, which exhibited considerable variability, and in the remaining surface loops.

After generating the mutant sequences, the mutant S proteins were expressed in *E. coli*, and the proteins were purified, refolded, and tested to determine whether they had altered biological activity (see Methods). Their ability to specifically inhibit S_1 pollen was compared with that of the nonmutated wild-type S_1 (S_1wt) recombinant protein. Figure 2 illustrates the normal S-specific activity of the S_1wt recombi-

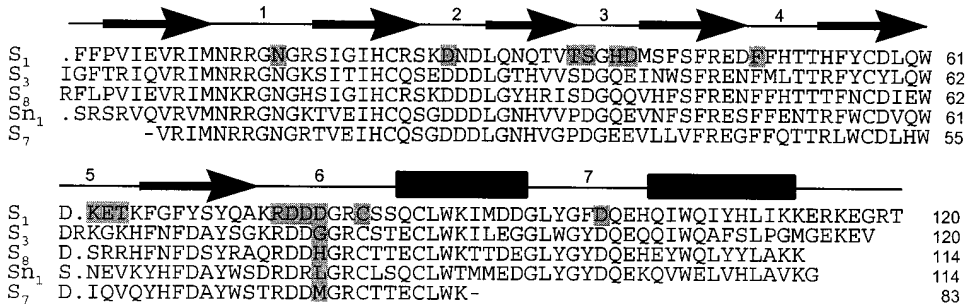

Figure 1. Alignment of the *P. rhoeas S* Alleles and Secondary Structure Predictions.

Shown is an alignment of the deduced amino acid sequences of the mature polypeptides of all *P. rhoeas S* alleles analyzed to date, including the partial S_7 sequence. Sequences for products of alleles S_1, S_3, S_7, and S_8 are from *P. rhoeas*; the Sn_1 product is from *P. nudicaule.* The numbers at the end of each line of the sequences indicate the amino acid residue positions at the end of that line. Above the sequences, the predicted secondary structure of all of these proteins is outlined. The arrows indicate predicted β strands, blocks indicate predicted α helices, and numbered lines 1 to 7 indicate the predicted hydrophilic surface loops. The only amino acid residue (Asp-79 in loop 6 of S_1) that is completely variable across these five sequences is indicated in the gray-shaded vertical block. The positions of mutations made in the S_1 protein are also indicated in gray blocks.

nant protein. This analysis reveals that at concentrations of ~10 μg mL^{-1} and above, complete S-specific inhibition of incompatible pollen can be observed. Furthermore, even at the highest concentration tested (150 μg mL^{-1}), no nonspecific inhibitory effect was detectable at these concentrations.

Both Variable and Conserved Residues in Loop 6 Participate in Inhibition of Incompatible Pollen

Figure 3A shows the biological activity of the mutant S proteins generated in loop 6. The first mutation made was D79G (substitution of Gly for Asp at position 79 in the S_1 product). This is a substitution in the S_1 sequence, with the residue found at this position in the S_3 protein. As shown in Figure 3A, D79G was unable to inhibit S_1 pollen, even when applied at 150 μg mL^{-1} (which is ~15-fold the amount of protein normally required to inhibit incompatible pollen). Thus, this single alteration in the sequence resulted in the complete loss of S-specific activity. This substitution did not confer S_3 inhibitory activity on the mutant.

A virtually identical result was obtained with D79H, which is the corresponding residue from S_8. Inhibition of S_1 pollen by D79H was only 4.3% that of inhibition by S_1wt, even at a concentration of 150 μg mL^{-1}. This was not statistically significantly different from the germination medium control ($P = 0.05$). We have also made the reciprocal mutation to the S_8 sequence by substituting the hypervariable His-80 in S_8 with Asp, which is located at the same relative position in the S_1 sequence. This mutation to the S_8 allele (H80D) resulted in a complete loss of biological activity toward S_8 pollen, as shown in Figure 3A, and did not confer inhibitory activity toward S_1 pollen. These data clearly demonstrate that substitutions of Asp-79 in the S_1 protein and the corresponding His-80 in the S_8 protein have a critical effect on the S-specific biological activity of the stigmatic S protein. These mutant S proteins are completely unable to inhibit pollen carrying the S_1 allele, that is, their ability to induce an incompatible response has been removed. Therefore, the amino acid residues at this hypervariable site in loop 6 must play a critical role in recognition and inhibition in the SI interaction of the stigmatic protein with incompatible pollen.

In contrast to the hypervariability seen at position 79 in S_1, several of the remaining residues in loop 6 (residues 76 to 78) are either completely or highly conserved across the five alleles that we have sequenced thus far (as shown in Figure 1). However, because ~65 *S* alleles are predicted to occur in *P. rhoeas* (Lane and Lawrence, 1993), it is quite possible that the amino acids that occupy these apparently conserved positions do, in fact, vary in certain alleles. Either way, their proximity to Asp-79 suggests that they may still contribute to the interaction between the S protein and the pollen component. On this basis, Arg-76 and Asp-77 and Asp-78 in S_1 were substituted in turn with His.

As illustrated in Figure 3A, R76H had no detectable effect on inhibition of S_1 pollen. This indicates that this residue does not participate in the recognition of incompatible pollen. Importantly, it also implies that the protein can tolerate substitutions to nonessential residues with no apparent effect on biological activity. In contrast, both D77H and D78H dramatically reduced the inhibitory activity of the protein against S_1 pollen. At best, the activities of D77H and D78H were only 6 to 9% of the activity exhibited by S_1wt and were not significantly different ($P = 0.05$) from the germination medium control. The double mutant protein D77H/D78H was completely inactive against S_1 pollen, and no pollen inhibitory activity was detected, as shown in Figure 3A.

Although the substitution of Asp with His could be considered relatively conservative in terms of hydration potential, it removed a negative charge (two in the case of D77H/D78H). To explore the significance of a negatively charged carboxyl side chain, we constructed a second double mutant (D77E/D78E). Glu is charged negatively like Asp but has a greater side chain volume. As shown in Figure 3A, when tested against S_1 pollen, no statistically significant difference ($P = 0.05$) was detected between the biological activities of D77E/D78E and S_1wt. These data suggest that the negatively charged carboxyl side chains of these two conserved, acidic amino acids are an important element in the recognition of S_1 pollen.

Effect of Mutations in Surface Loop 5 of the S_1 Protein

The sequence alignment also revealed a number of variable amino acids in loop 5 (see Figure 1). In particular, S_3 possesses an extra amino acid residue at this point. Initially, Lys-63 in S_1 was substituted with the residue found in the corresponding position in S_3 to give construct K63R. As shown in Figure 3B, when this mutant was assayed for biological activity, it was fully active, displaying 102% activity of that exhibited by S_1wt when tested at 25 μg mL^{-1}. Because there was no significant reduction of biological activity, we substituted all three variable residues in loop 5 with corresponding S_3 residues. This triple mutant K63R/E64K/T65G retained close to full activity when applied to pollen at concentrations >75 μg mL^{-1} (83% that of S_1wt at 75 μg mL^{-1}). However, as shown in Figure 3B, at 25 μg mL^{-1}, biological activity was significantly reduced ($P = 0.05$) to 58% of S_1wt. This reduction suggests that although residues in loop 5 of the S_1 protein may influence the activity of the protein, they are not solely responsible for determining *S* allele specificity of the protein. It is possible that the loss of activity is simply attributable to these substitutions having a nonspecific adverse influence on protein refolding. Although this explanation cannot be ruled out, it is considered unlikely for two reasons. First, the residues are substituted with corresponding residues from the S_3 protein sequence, and second, they are located in a hydrophilic surface loop, where they are less likely to have a detrimental effect on protein folding.

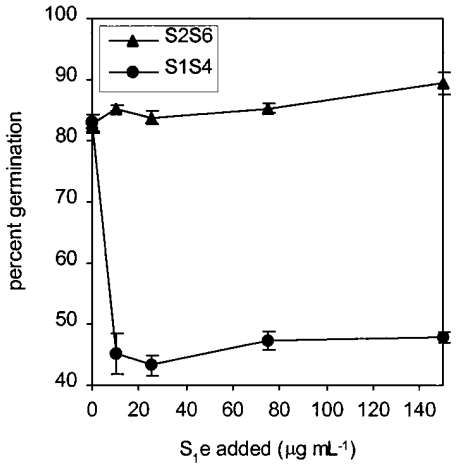

Figure 2. A Dose–Response Curve for the S-Specific Inhibition of S_1 Pollen by the S_1wt Protein.

The S-specific inhibition activity of the recombinant S_1wt protein was determined using S_1S_4 and S_2S_6 pollen. The biological activity of the S protein was tested over a range of concentrations from 10 to 150 μg mL^{-1}. Pollen from plants of the S_1S_4 genotype was inhibited by ~50% (which is the theoretical maximum inhibition because the pollen used was heterozygous) at concentrations ≥10 μg mL^{-1}. In contrast, fully compatible pollen (from plants of genotype S_2S_6) revealed no detectable inhibition, even at the higher concentrations of S_1wt used. This clearly demonstrates that the S-specific inhibitory effect of the S_1wt protein is effective in the range of 10 to 150 μg mL^{-1}, with no nonspecific inhibitory effect at these concentrations.

The Majority of Mutations in Surface Loops Have No Biological Effect

To further explore a role in pollen recognition for residues within the remaining predicted loops (loops 1, 2, 3, 4, and 7), we constructed a series of single and double mutations to the S_1 sequence, targeting each loop sequentially: in loop 1, N15H; in loop 2, D27H; and in loop 3, two double mutants were made. Thr-36, which is predicted to lie at the edge of the loop, was substituted for Ser, and Ser-37 was substituted for Asp (T36S/S37D); in addition, H39Q/D40E was created. Loop 4 differed from the other surface loops in that the secondary structure prediction suggested that the two conserved Phe residues located within this loop may produce a shallow hydrophobic pocket. To test the significance of this, we substituted Phe-49 for Met (F49M), which has similar hydrophobicity but differs significantly in structure, because it does not possess an aromatic side chain. In loop 7, Asp-99 was substituted for His (D99H).

The mutant S proteins with substitutions in loops 1, 2, 3, 4, and 7, when tested for their ability to specifically inhibit S_1 pollen, were demonstrated to be biologically active, as shown in Figure 3B. When tested at a concentration of 75 μg mL^{-1}, they all exhibited activity against S_1 pollen indistinguishable from S_1wt. With the exception of the loop 2 mutant (D27H), the result was essentially the same at a concentration of 25 μg mL^{-1}. Mutant D27H, however, did exhibit a significant reduction ($P = 0.05$) in activity to ~78% that of S_1wt. This observation is reminiscent of that found for the loop 5 triple mutation, and for the same reasons, its significance is unclear. Overall, the mutations presented in this section had a minor or no effect on the ability of the S_1 stigmatic protein to inhibit S_1 pollen. On the basis of our data, the S_1 protein can tolerate mutations in these remaining surface loops without detriment to its biological activity.

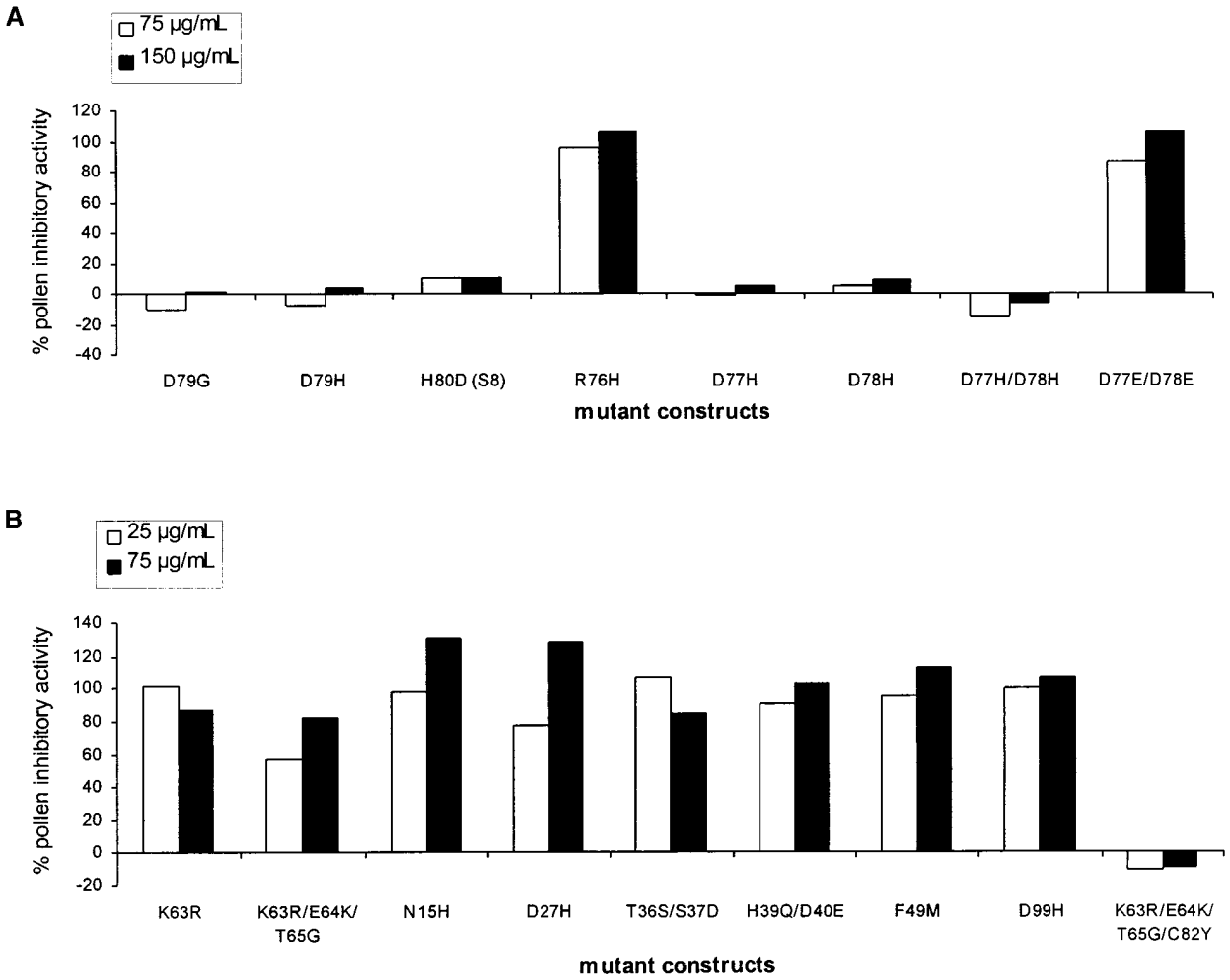

Figure 3. Effect of Mutations in the S_1 Protein on S-Specific Pollen Inhibitory Activity.

The mutant S proteins were tested for their ability to specifically inhibit S_1 pollen. The normal S-specific activity of the wild-type S_1 (S_1wt) recombinant protein served as the standard against which the activity of the mutant S proteins was compared. The mutant proteins were routinely assayed at several concentrations in a randomized format, in at least three independent experiments, and against pollen carrying the S_1 allele and fully compatible pollen of a different *S* genotype (see Methods). The activity of the mutant S proteins is expressed as the percentage of activity relative to S_1wt.

(A) Effect of loop 6 mutations. Shown is pollen inhibitory activity with substitutions to the predicted loop 6 region of the S_1 amino acid sequence. Pollen inhibitory activity of the mutant proteins is shown for two concentrations (75 and 150 $\mu g\ mL^{-1}$) and is expressed as a percentage of S_1wt protein activity. Note that all mutations, except for R76H and D77E/D78E, result in complete loss of pollen inhibitory activity.

(B) Effect of other mutations. Shown is pollen inhibitory activity with substitutions to the predicted hydrophilic loops of the S_1 amino acid sequence. Pollen inhibitory activity of the mutant proteins is shown for 25 and 75 $\mu g\ mL^{-1}$ and is expressed as a percentage of S_1wt protein activity. Note that there is no significant reduction in pollen inhibitory activity, except for K63R/E64K/T65G and K63R/E64K/T65G/C82Y.

A Conserved Cys Residue Is Essential for Activity of S_1

A notable feature of the poppy S protein sequence alignment is the presence of four absolutely conserved cysteine residues, relative to positions 23, 57, 82, and 86 of the S_1 sequence (see Figure 1). We previously suggested that these may form two disulfide bridges that are essential in maintaining tertiary structure (Walker et al., 1996; J.P. Ride, unpublished results). To investigate the importance of these conserved Cys residues, we introduced a further mutation into the loop 5 triple mutant K63R/E64K/T65G/C82Y. As shown in Figure 3B, this substitution (C82Y) caused complete inactivation of the protein, and no S_1 pollen inhibition activity was detectable. Because this mutant has virtually

normal activity, we interpret this result to implicate Cys-82 as being of vital importance to the activity of the protein. This is most probably due to its influence on the tertiary structure of the S protein.

DISCUSSION

In the studies described here, we have begun to define the role of regions in the stigmatic S proteins of *P. rhoeas* that are required for allele-specific inhibition of incompatible pollen. Our studies indicate that variability in amino acid sequence between stigmatic S proteins is likely to be responsible for determining *S* allele specificity rather than glycosylation. This is based on two key observations. First, not all poppy S proteins, for example, S_3 (Walker et al., 1996), are glycosylated. Second, there is no detectable difference in biological activity between the S_1 protein extracted from poppy stigmas (which is glycosylated) and the nonglycosylated recombinant S protein expressed in *E. coli* (Foote et al., 1994; Franklin-Tong et al., 1995).

Residues Located in Predicted Hydrophilic Loop 6 Participate in Pollen Recognition

In contrast to the S RNases of the Solanaceae (Ioerger et al., 1990, 1992), the sequence alignments between the poppy S proteins do not readily identify defined hypervariable domains. Instead, short blocks of identity are interspersed with variable residues. The available sequence information from the cloned Papaver *S* alleles (Foote et al., 1994; Walker et al., 1996; Kurup et al., 1998) suggests that it is highly probable that residues determining allele specificity will be scattered throughout the primary sequence.

Only one amino acid site has been identified that is polymorphic across available *S* alleles sequenced thus far. This site is Asp-79 in the S_1 protein and the corresponding His-80 in the S_8 protein (Foote et al., 1994; Walker et al., 1996; Kurup et al., 1998). This site is predicted to lie within a hydrophilic loop (loop 6) and, on the basis of the current evidence, appears to be flanked by conserved, charged amino acids. The studies described here demonstrate that mutation of this site resulted in the complete loss of biological activity. This clearly implies that the amino acid found in the different S proteins at this site plays a crucial role in pollen recognition in these alleles; because it is variable between *S* alleles, it may participate in defining allelic specificity. However, although the ability to reject S_1 pollen can be affected by mutation of this single residue, it cannot be the sole determinant of *S* allele specificity for two key reasons. First, the mutations in S_1, D79H (a substitution from S_1 to S_8), and H80D (a substitution from S_8 to S_1) did not confer a new allelic specificity on either mutant protein (data not shown). That is, simply switching the S_1 residue at this site for its S_8 counterpart, and vice versa, did not change specificity. Second, population genetics studies of SI in *P. rhoeas* predict that there are ~65 *S* alleles in the species (Lane and Lawrence, 1993). Because there are only 20 amino acids, allelic variation cannot be specified by a single polymorphic site within the protein. It would seem likely that specificity is determined by several residues that, in combination, specify an allelic identity.

Despite introducing a series of single, double, and triple mutations into the remaining predicted surface loops, we have detected no dramatic loss of S protein inhibitory activity comparable with that seen with the loop 6 mutations. This clearly demonstrates that mutations to these residues do not affect S-specific biological activity. Therefore, we conclude that in the case of S_1, these residues do not contribute to the *S* allele–specific recognition of the pollen. Our results also illustrate that the S proteins can tolerate mutations within these regions and that alterations at these sites presumably do not have any significant effect on protein folding.

The importance of maintaining the integrity of the tertiary structure is clearly illustrated by the mutation to one of the four conserved cysteine residues. When Cys-82 was replaced with a Tyr residue (C82Y), the mutant protein was totally inactive. Although there is as yet no direct evidence, we believe that Cys-82 forms a disulfide bridge with Cys-86, whereas Cys-24 and Cys-57 form another disulfide bridge. This hypothesis is based on the sequence alignments of the recently identified gene family of S protein homologs (*SPH*) in Arabidopsis (J.P. Ride, F.C.H. Franklin, and D.F. Marshall, unpublished data). To date, 17 *SPH* sequences have been identified. These all encode small (~15 kD), basic, secreted proteins that are predicted to adopt secondary structures that are very similar to those of the S proteins. All SPH sequences possess two Cys residues that align with Cys-24 and Cys-57 of S_1. This strongly suggests that this pair of Cys residues forms one of the two predicted disulfide bridges in the poppy S proteins. If this is the case, then Cys-82 and Cys-86 potentially would form a short hairpin structure adjacent to loop 6. The loss of pollen inhibitory activity caused by the substitution of one of these residues (e.g., C82Y) suggests that this hairpin structure may itself participate in pollen recognition.

Further evidence that amino acid residues in loop 6 play an important role in pollen recognition and the interaction of the S proteins with incompatible pollen was revealed by the mutation of the Asp residues at positions 77 and 78 in loop 6. These residues are conserved across all poppy *S* alleles characterized to date. In the case of S_1 at least, it seems that the negative charges present in the carboxyl side chains of Asp-77 and Asp-78 are of particular importance. The evidence is that these residues can be replaced with equally acidic Glu residues with little or no effect on biological activity, whereas substitution with more basic His residues effectively abolishes function. It is conceivable that these charged amino acids stabilize the interaction with the

pollen component or perhaps have a role in maintaining the structural integrity of the S_1 protein in the region of loop 6.

An important goal of future studies is to determine the tertiary structure of an S protein. This will enable the identification of amino acids located at the protein surface or at other positions that are available for interaction with the pollen component. It will also identify amino acids that may be at distant positions in the primary sequence but that are juxtaposed to the same region when the protein adopts its active conformation. Such information is likely to be essential if we are to define precisely which amino acid residues specify a particular allelic identity of an S protein. It may also provide some explanations for the minor reductions in activity that we have observed with some of the mutant proteins described. It is possible that in the tertiary form of the S protein, these residues are located close to key amino acids and that the mutations have had an indirect, adverse effect on the ability of crucial residues to interact with the pollen component.

These studies have enabled us to define several residues within the stigmatic S_1 protein that are required for allele-specific inhibition of S_1 pollen. Furthermore, they have enabled us to rule out the participation of several other residues. Taken together, these data strongly indicate that the region of the S_1 protein corresponding to surface loop 6 is a key element in the recognition of incompatible pollen.

METHODS

Plant Material and Cross-Classification of *S* Alleles

The families of the Shirley variety of *Papaver rhoeas* were as previously described by Foote et al. (1994). Plants carrying *S* alleles distinct from S_1, S_3, and S_8 were identified using a combination of cross-classification by pollination (see Campbell and Lawrence [1981] and Lane and Lawrence [1993] for full experimental details) and protein gel blotting with immunodetection using antisera raised to recombinant S_1 and S_3 proteins.

Site-Directed Mutagenesis

The wild-type S_1 protein (S_1wt) was originally produced from cells carrying the vector pPRS100 as described by Foote et al. (1994). Two methods were used to introduce amino acid substitutions into the S_1wt protein. Specific mutations were introduced into loop 6 of the S_1 amino acid sequence by using the polymerase chain reaction–based method described by Higuchi et al. (1988). DNA amplification was performed using mismatched primers to incorporate the required mutation from a DNA template of the gene for the mature S_1 polypeptide ligated into pBluescript SK+ (Stratagene, La Jolla, CA). The mutated gene was then cloned into the expression vector pMS119 (Lessl et al., 1992) at the NdeI and HindIII restriction sites. Mutations to other loops were performed by the method described by Stanssens et al. (1989) using the S_1 gene cloned into the pMac5-8 phagemid vector via the BamHI and HindIII restriction sites. The mutations were incorporated using mismatched primers and the mutated gene cloned into the vector pMS119, as given above. To facilitate protein expression, a translation initiation codon was incorporated at the start of the gene sequences.

Nucleotide Sequencing

Nucleotide sequencing was performed both manually by the dideoxy method (Sanger et al., 1977) using the T7 Sequenase 2.0 kit (Amersham) and by automated sequencing (Alta BioScience, University of Birmingham, UK).

Database Searches and Structural Predictions

For preliminary sequence analysis, we used software from the Genetics Computer Group (Madison, WI). More detailed comparisons were performed with MACAW using BLOSUM 62 comparison tables (Schuler et al., 1991; Lawrence et al., 1993). The predicted hydrophilicity plots for S_1 and S_3 were determined according to the method of Kyte and Doolittle (1982) using a window of seven residues. Secondary structures of the S proteins were predicted using the PHD program (Rost and Sander, 1993, 1994; Rost et al., 1994).

Expression of Recombinant Proteins

Cells containing wild-type and mutant gene constructs were induced with 1 mM isopropyl β-D-thiogalactopyranoside for 4 hr, resulting in high-level expression of recombinant proteins that accumulated as insoluble inclusion bodies. Cells were harvested by centrifugation (5000*g* at 4°C for 10 min) and resuspended in lysis buffer containing 50 mM Tris-Cl, 100 mM NaCl, and 1 mM EDTA, pH 8.0. They were disrupted by using lysozyme and deoxycholate and by sonication: inclusion bodies were collected by centrifugation and washed in lysis buffer (Sambrook et al., 1989). The resulting proteins were dissolved in lysis buffer containing 6 M guanidine–HCl, 0.5 M 2-mercaptoethylamine, and 0.125 mM phenylmethylsulfonyl fluoride, pH 8.0, for 1 hr at room temperature and refolded by rapid dilution in 100 mM Tris containing 50 mM L-arginine, 2 mM EDTA, 10 mM cystamine, and 5% (v/v) glycerol, pH 8.0, at 14°C to a final concentration of 100 μg mL^{-1}. After incubation overnight, the solution was dialyzed against 50 mM Tris-HCl and 100 mM NaCl, pH 8.0, and concentrated against the same buffer containing 12.5% polyethylene glycol 6000. The final protein solution was stored at −80°C. Protein concentration was estimated using the Coomassie Brilliant Blue R 250 dye-binding method (Bradford, 1976).

Testing the Biological Activity of the Mutant S Proteins

The biological activity of mutant proteins was determined using an adaptation of our in vitro bioassay (Franklin-Tong et al., 1988; Foote et al., 1994). Recombinant mutant proteins were assayed at several concentrations based on the dose–response curve. The concentrations used for testing the biological activity of the mutant protein were significantly above levels required to detect S-specific pollen inhibition activity to ensure that lack of activity could not be attributed to using a concentration that might be close to the threshold of activity. Protein activity was initially tested at 25 and 75 μg mL^{-1} if reduced or no activity was detected. Additional tests were performed

at 75 and 150 μg mL^{-1}, which are concentrations significantly above levels required to detect S-specific pollen inhibition by S_1wt. To detect any nonspecific inhibition, we also tested proteins against pollen of a fully compatible *S* genotype.

To assay for biological activity, we dialyzed proteins against liquid germination medium containing 1% (w/v) H_3BO_3, 1% (w/v) KNO_3, 1% (w/v) $Mg(NO_3)_2 \cdot 6H_2O$, 3.1% (w/v) $CaCl_2 \cdot 2H_2O$, and 13.5% (w/v) sucrose. Half-compatible pollen carrying the S_1 allele (e.g., S_1S_4 or S_1S_3) and fully compatible pollen carrying S_2S_4 or S_4S_6 alleles were allowed to prehydrate on multiwell slides for 30 min at 25°C. Aliquots (20 μL per well) of germination medium and appropriately diluted S proteins were applied to the wells, and pollen was allowed to germinate for 2 hr at 25°C. Experiments were randomized on the slides and included three replicates of each treatment. Wells were scored for germinated and nongerminated pollen ($n > 100$ per well). The percentage of germination of pollen that was treated with germination medium (no protein) and S_1wt and the mutant S protein was calculated. Data plotted represent the relative activity of the mutant S proteins expressed as a percentage of S_1wt activity. Minitab 10.51 Xtra (Minitab Inc., State College, PA) was used to perform a one-way analysis of variance of the percentage of germination after angular transformation of the data.

ACKNOWLEDGMENTS

We thank Antony Jones for technical assistance and John Martin and colleagues for horticultural support. This research was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) and Japan Society for the Promotion of Science (J.S.P.S.).

Received June 17, 1998; accepted August 5, 1998.

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