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Identification of the pollen self-incompatibility determinant in *Papaver rhoeas*

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Abstract

Higher plants produce seed through pollination, using specific interactions between pollen and pistil. Self-incompatibility (SI) is an important mechanism used in many species to prevent inbreeding, and is controlled by a multi-allelic *S* locus1,2. "Self" (incompatible) pollen is discriminated from "non-self" (compatible) pollen, by interaction of pollen and pistil *S* locus components, and is subsequently inhibited. In *Papaver rhoeas*, the pistil *S* locus product is a small protein that interacts with incompatible pollen, triggering a Ca^{2+}-dependent signalling network, resulting in pollen inhibition and programmed cell death3-7. Here we have cloned three alleles of a highly polymorphic pollen-expressed gene, *PrpS*, from *Papaver* and provide evidence that this encodes the pollen *S* locus determinant. *PrpS* is a single copy gene linked to the pistil *S* gene, *PrsS*. Sequence analysis indicates that *PrsS* and *PrpS* are equally ancient and are likely to have co-evolved. *PrpS* encodes a novel ~20 kDa protein. Consistent with predictions that it is a transmembrane protein, PrpS is associated with the plasma membrane. We show that a predicted extracellular loop segment of PrpS interacts with PrsS and, using *PrpS* antisense oligonucleotides, we demonstrate that PrpS is involved in *S*-specific inhibition of incompatible pollen. Identification of *PrpS* represents a major advance in our understanding of the *Papaver* SI system. As a novel cell-cell recognition determinant it contributes to the available information concerning the origins and evolution of cell-cell recognition systems involved in discrimination between "self" and "non-self", which also include histocompatibility systems in primitive chordates and vertebrates.

Keywords

Self-incompatibility; pollen *S* receptor; *Papaver rhoeas*; pollen tube inhibition

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It has been established that self-incompatibility (SI) has evolved independently several times. Three SI systems have been well characterised at a molecular level1,2. Both pollen and pistil *S* determinants are expected to have co-evolved and be physically linked to the *S* locus in order to maintain a functional SI system. Other characteristics expected of them are high levels of allelic polymorphism and tissue specific expression. Most importantly, they

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Author information *PrpS1* and *PrpS8* sequences have been deposited in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) as accessions AM743176, AM743177 and FN178511.
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should function in mediating the SI response. To fully understand how different SI systems operate, identification of both the pistil and pollen *S* locus components, together with establishing mechanisms involved in pollen inhibition is crucial. Previously, we identified the pistil *S* determinant for *Papaver rhoeas*8-10 and established several components involved in pollen inhibition3-7,11. Although we identified a glycoprotein in pollen that bound to the pistil S protein, studies indicated that it was not the pollen *S* determinant, though it might modulate the SI response12. Recent analysis of the S_1 locus enabled identification of the pollen component of the *S* locus on a cosmid clone comprising a 42 kb region at the S_1 locus.

Nucleotide sequencing and analysis identified a novel putative open reading frame (ORF) 457 bp from the S_1 pistil gene (Fig 1a). Expression analysis using RT-PCR revealed that the ORF was specifically transcribed in pollen (Fig 1b), appearing during anther development (Fig 1c). The temporal expression pattern is very similar to that of the pistil *S* gene8. These data suggested that this ORF was a candidate for the *Papaver* pollen *S* gene (designated *PrpS: Papaver rhoeas pollen S*). We propose renaming the gene that determines SI specificity in pistils (currently designated simply *S*) to provide a clearer nomenclature; we suggest *PrsS* (for *Papaver rhoeas* stigma *S* determinant).

The cDNA of $PrpS_1$ comprises 1206 bp containing a coding region of 579 bp encoding a 192 amino acid polypeptide with a predicted M_r of 20.5 kDa, pI 7.55. We subsequently cloned $PrpS_3$ and $PrpS_8$ from S_3S_8 pollen RNA. The $PrpS_3$ and $PrpS_8$ coding sequences are 576 bp and 582 bp (191 and 193 amino acids) respectively (Fig 1d); $PrpS_3$ and $PrpS_8$ encode proteins of predicted Mr of 21.1 kDa (pI 6.57) and 20.9 kDa (pI 8.51) respectively. Southern blotting revealed that *PrpS* is single copy (Supplemental Fig 1), so the related sequences identified as $PrpS_3$ and $PrpS_8$ are clearly allelic to $PrpS_1$, rather than being related/ paralogous genes.

Segregation analyses were conducted to obtain evidence of genetic linkage at the *S* locus between $PrsS_3$ and $PrpS_3$ and between $PrsS_8$ and $PrpS_8$. Specific primers were used to amplify regions of the pistil $PrsS_1$, $PrsS_3$ and $PrsS_8$ and pollen $PrpS_1$, $PrpS_3$ and $PrpS_8$ genes from genomic DNA from two full-sib families segregating for these haplotypes (45 and 25 individuals, a total of 140 *PrpS*/*PrsS* pairs). $PrpS_1$ was amplified only from plants carrying S_1; $PrpS_3$ was amplified only from plants carrying S_3 and $PrpS_8$ was amplified only from plants carrying the S_8 haplotype. The pistil $PrsS_1$, $PrsS_3$ and $PrsS_8$ sequences were also amplified only from plants carrying the respective *S* haplotypes (Fig 1e), as expected. This demonstrates co-segregation and linkage of $PrpS_1$, $PrpS_3$ and $PrpS_8$ and their cognate *PrsS* genes, as no recombination was detected (recombination frequency <0.021). Thus, at the 95% rejection level we can be confident that there is no recombination.

A strikingly high level of allelic sequence polymorphism is a well-documented feature of *S* locus proteins; *S* alleles have unusually high amino acid sequence divergence within species13-15. *Papaver* is no exception; the pistil proteins $PrsS_1$ and $PrsS_3$ exhibit 46% sequence divergence; $PrsS_1$ and $PrsS_8$ have 40%, and $PrsS_3$ and $PrsS_8$ 46% divergence. PrpS proteins exhibit a similar level of polymorphism (Fig 1d); the $PrpS_1$ and $PrpS_3$ predicted amino acid sequences are 50% divergent; $PrpS_1$ and $PrpS_8$ exhibit 40% divergence; $PrpS_3$ and $PrpS_8$ are 47% divergent16.

The pollen and pistil *S* determinants should exhibit evidence of co-evolution. Examination of the *PrpS* sequences for non-synonymous to synonymous (*Ka/Ks*) substitutions reveal that the *PrsS* alleles have a mean *Ka/Ks* ratio of 0.234, and the *PrpS* alleles have a mean *Ka/Ks* ratio of 0.368 (Supplemental Table 1). A two-tailed *t*-test showed no significant difference

between substitution rates in *PrpS* and *PrsS* genes. These data suggest that the pollen and pistil *S* alleles co-evolved and are likely to be similarly ancient.

PrpS has no significant sequence homology to any protein in existing databases. Sequence analysis, using a range of prediction programmes, indicated that PrpS has 3-5 predicted transmembrane helices and alignment of the three *PrpS* alleles indicates that they share a similar topology (Fig 1d; Supplemental Fig 2). In support of predictions that PrpS is a transmembrane protein, western analysis using antisera raised against $PrpS_1$, revealed that $PrpS_1$ was detected as a ~20 kDa protein specifically in S_1 pollen membrane-enriched extracts (Fig 2a-c). Moreover, immunolocalization studies revealed that $PrpS_1$ is associated with the pollen tube plasma membrane (Fig 2d). Although the PrpS sequences do not show any particular bias for the "positive inside rule"17, structural predictions suggest an extracellular loop segment, comprising amino acids ~60-100 (63-97 using TMHMM, Fig 1d; Supplemental Fig 2). We hypothesized that this region might be involved in the interaction with PrsS and show that a peptide corresponding to part of the predicted PrpS extracellular loop interacted with the PrsS protein, while the corresponding randomized peptide did not (Fig 2e).

To determine whether PrpS is functionally involved in the SI response, we investigated whether it mediates *S* specific pollen inhibition, using *in vitro* SI bioassays8. Peptides based on extracellular domains of receptors have been used to identify ligand-binding epitopes *via* their ability to block the receptor-ligand interaction18. Preliminary experiments with the peptide used in the binding assay tested if it could block SI-mediated inhibition. Pollen from plants with haplotypes S_1S_3, when challenged with incompatible recombinant PrsS, were rescued from inhibition by $PrpS_1$ peptides (n = 6; P < 0.001, ***), while randomized peptides based on the same amino acids had no effect (n=3; N.S.; Supplemental Fig 3). This is consistent with the hypothesis that this region is involved in recognition and indicated that PrpS might mediate pollen inhibition. To confirm this possibility, we used an antisense oligonucleotide approach3,19. We hypothesized that if PrpS functions as the pollen *S* determinant, knockdown of its expression should result in alleviation of pollen tube inhibition in an *S*-specific manner. We induced SI *in vitro* in the presence of either antisense- (as-ODNs) or sense- (s-ODNs) oligonucleotides to test this hypothesis. As our plants are heterozygous for *S* haplotypes, the pollen phenotype of plants with S_1S_3 haplotypes should theoretically be 50% S_1 and 50% S_3. Thus, if the interaction is *S*-specific, as-ODNs specific for $PrpS_1$ should only affect the 50% of pollen (carrying S_1).

SI induced strong inhibition of pollen tube growth (a 79% reduction in length compared to the controls) and we observed a significant alleviation of this inhibition in an incompatible combination in the presence of as-ODNs and not with corresponding s-ODNs (Fig 3). With pollen from plants with S_1S_3 haplotypes, SI induced strong inhibition of pollen tube length (22.1%, P<0.001 ***, n=300) and addition of as-$PrpS_1$-ODNs gave a highly significant recovery of SI-treated tubes (58.3% increase in length compared to SI-treated; P<0.001, ***, n=150), and incompatible pollen responded in a bimodal manner, consistent with only S_1 pollen being affected (Supplemental Fig 4). When as-$PrpS_8$-ODNs were added to the same pollen from plants with S_1 and S_3 haplotypes, they did not alleviate SI-induced inhibition (P=0.604, N.S., n=150). This demonstrates that the $PrpS_1$ and $PrpS_8$ as-ODNs had an *S*-specific effect. As expected, s-$PrpS_1$-ODNs did not affect the SI response (P=0.591, N.S., n=150).

To further confirm the *S*-specific effect of the as-ODNs, we also tested their effect on pollen from plants with haplotypes S_3S_8. SI resulted in inhibited pollen tubes (19.8% of the control, n=300), and addition of as-$PrpS_8$-ODNs alleviated the SI-induced inhibition, giving a highly significant 100.3% increase in pollen tube length (P=<0.001, ***, n=150), whereas there

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was no effect using as-*PrpS*$_1$-ODNs (P=0.336, N.S., n=150) or s-*PrpS*$_8$-ODNs (P=0.565, N.S., n=150) (Fig 3). Together these data demonstrate that PrpS plays a crucial role in SI-induced *S* haplotype-specific pollen tube inhibition.

In summary, we have cloned a polymorphic pollen-expressed gene, *PrpS*. Together our data are consistent with the hypothesis that PrpS is the *Papaver* pollen *S* determinant as it mediates *S*-specific recognition and inhibition. SI in *Papaver* is distinct from the other well characterized SI systems (i.e. from both the *Brassica* pollen *S* determinant SCR/SP11 and the pollen F-box protein SLF/SFB from the S-RNase based SI system)[1,20-23]. As *PrpS* has no homologues, its nature is intriguing. Self-nonself discrimination and other recognition systems which are controlled by a highly polymorphic locus are not limited to SI; other systems include disease resistance in plants[24], and histocompatibility systems in animals[25-27]. These parallels between non-analogous recognition systems were recognized, and their importance appreciated, long before the molecular basis of these systems were elucidated[25] and the nature of their polymorphism has intrigued population and evolutionary biologists for decades. The identification of PrpS as a novel cell-cell recognition determinant thus contributes to the available information regarding the evolution of self-nonself recognition systems.

METHODS SUMMARY

Cloning of *PrpS*$_1$, *PrpS*$_3$ and *PrpS*$_8$

A genomic clone of *PrpS*$_1$ was identified by nucleotide sequence analysis of a 42 kb clone carrying the S_1 locus, obtained by screening a *Papaver rhoeas* S_1S_3 cosmid genomic DNA library (SuperCos1, Stratagene) with *PrsS*$_1$ cDNA. The DNA upstream and downstream of the *PrsS*$_1$ gene was sequenced and analyzed using BLAST (http://ncbi.nlm.nih.gov/BLAST) and ORF Finder (http://searchlauncher.bcm.tmc.edu)[28]. The organisation of *PrpS*$_1$ and *PrsS*$_1$ genes was confirmed using PCR on genomic DNA of S_1- and non-S_1- containing plants. *PrpS*$_3$ and *PrpS*$_8$ cDNAs were obtained using RT-PCR, 3′ and 5′-RACE PCR (see **On-line Methods** for primer details) on pollen cDNA from suitable *S*-haplotypes, using low annealing temperatures (48 °C).

Ka/Ks calculations

DNAsP[29] was used to estimate the *Ka* (the number of non-synonymous substitutions per non-synonymous site), and *Ks* (the number of synonymous substitutions per synonymous site) for pairs of *PrsS* and *PrpS* nucleotide sequences.

Peptide binding assay

A 15 aa peptide (DQKWVVAFGTAAICD) corresponding to part of the predicted extracellular loop segment of PrpS$_1$ (TMHMM; http://www.cbs.dtu.dk/services/TMHMM [30]) and a randomized version (FTVDVKDCAAAWGQI) were synthesized (Alta Bioscience, University of Birmingham). The peptides (10 μg, 1 μg, 0.1 μg) were bound to PVDF membrane. This was incubated with recombinant PrsS$_1$ and then probed for binding using α-PrsS$_1$ antisera and alkaline phosphatase detection.

Antisense oligo silencing of *PrpS* expression

Phosphorothioated gene-specific antisense oligodeoxynucleotides (as-ODN) and their sense controls (s-ODN) were designed (*PrpS*$_1$ as-ODN: gtccTCCCAGTATTAttga, *PrpS*$_1$ s-ODN: tcaaTAATACTGGGAggac, *PrpS*$_8$ as-ODN: ttccCACCAGCACAGCaatt, *PrpS*$_8$ s-ODN: aattGCTGTGCTGGTGgaa; lower case letters indicate bases linked by phosphorothioate bonds). Pollen was grown *in vitro* and pre-treated with as-ODNs and s-ODNs[3,19] for 1 h

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prior to induction of SI with recombinant $PrsS_1$, $PrsS_3$ and $PrsS_8$8. After 2 hours, pollen tubes were fixed in 2% formaldehyde and 150 pollen tube lengths were measured in three independent experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Methods

Sequence homology comparisons

Sequence identities were calculated for *PrsS* and PrpS, using pairwise comparisons of the amino acids comprising the mature peptide, using BLAST (BLOSUM62)31.

Southern blotting

Southern blots of *Papaver* genomic DNA from plants of different *S*-haplotypes (S_1S_8, S_1S_3, S_3S_8, S_2S_4, S_3S_4, S_7S_x) digested with *Eco*RV, *Bam*HI, *Pst*1 *or Bgl*II, were probed with a $PrpS_1$ probe. Blots were hybridised at 60°C overnight and washed down to 0.5 × SSC at 50°C, and signals detected using autoradiography.

Structural predictions for PrpS and interpretation of the data

We analysed PrpS sequences using a number of protein prediction programmes: TMHMM2.032, PredictProtein, SOSUI, HMMTOP, TMpred, TM-Finder, SPLIT 4, ConPredII, Phobius. TMHMM2.0 in particular, is regarded as a very robust transmembrane helix predictor, though predicting the number of membrane-spanning regions remains difficult33. Although predictions differ, they all predict that PrpS has transmembrane helices. We have been advised that the predictions suggest anything between 3-5 transmembrane segments and the alignment of the PrpS sequences suggests that all three proteins share a similar topology. Although many predictions indicated 3 or 5 TM domains, it is more likely that PrpS has four transmembrane segments, as it is a good number to make a four helix bundle in the membrane, as 3 and 5 TM proteins are rare (*Dr. A. Lovering, personal communication*). All the predictions indicate an extracellular loop segment. SI Fig 2 shows a cartoon indicating a prediction for the regions; this is a tentative assignment of possible topology.

Linkage analysis

Segregation analysis was carried out on individual plants from several full-sib families which share three *S* haplotypes and segregate for haplotypes S_1S_3 or S_3S_8 and S_1S_8 or S_3S_8. These are well established families, in which *S* haplotypes were designated ~20 years ago, produced using controlled crosses between individuals which had their *S*-haplotypes verified using aniline blue microscopy; they have been analyzed for *S* allele segregation for at least seven generations in a pedigree going back to 1994.

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The number of plants required to be analyzed, in order to statistically be confident at 95% rejection level34 that there is recombination, is 25 plants (one cannot formally provide evidence that there is never recombination). We carried out analysis of a full-sib family, segregating for pollen *PrpS*$_1$ and *PrpS*$_3$ and pistil *PrsS*$_1$ and *PrsS*$_3$ alleles (25 plants, 50 alleles) and for the S_1 and S_8 loci, we analysed 45 full-sib plants (90 alleles) to show segregation of pollen *PrpS*$_1$ and *PrpS*$_8$ and pistil *PrsS*$_1$ and *PrsS*$_8$. Thus, we examined segregation of a total of 140 *PrpS*/*PrsS* pairs of alleles. Genomic DNA from leaf tissue was extracted (Extract-N-Amp™ Plant PCR kit, Sigma-Aldrich) and gene specific primers used to specifically amplify *PrsS*$_1$, *PrsS*$_3$, *PrsS*$_8$, and *PrpS*$_1$, *PrpS*$_3$ and *PrpS*$_8$ sequences. **See SI Table 3 for primer details**. No recombination was detected (RF <0.021). Thus, at the 95% rejection level, we can be confident that there is no recombination.

RT-PCR to show tissue and developmental specificity

Standard RT-PCR techniques were used for expression analysis. Total RNA was extracted from anthers from plants with the S_1 haplotype at different stages of development and from different tissues (RNAeasy plant mini kit, QIAGEN) and cDNA synthesised (Omniscript RT kit, QIAGEN). Gene-specific primers (see **SI Table 3**) were used to amplify *PrpS*$_1$ transcripts; primers for the *P. rhoeas* glyceraldehyde-3-phosphate dehydrogenase (GAPD) gene acted as controls.

Production of antisera

The predicted 60 amino acid C-terminus of *PrpS*$_1$ (designated PrpS1-60C) was expressed as a HIS-tagged recombinant protein using pET21b (Novagen). Recombinant protein was isolated from *E. coli* BL21 (DE3) using Ni-NTA resin following the manufacturer's (QIAGEN) protocol. Antisera (PrpS1-60C) was raised in rats (ISL Immune Systems, Paignton, UK).

Protein extraction for SDS-PAGE and Western blotting

Extracts enriched for membrane proteins were made in 100 mM Tris-HCl pH 8, 200 mM NaCl, 2 mM EDTA, 1 M sucrose, 0.5% Triton X-100, Protease Inhibitor cocktail (Roche). Protein concentrations were determined35 and proteins separated using SDS-PAGE and electroblotted (400 mA, 3 h) onto Hybond C membranes (GE Healthcare). These were incubated with the PrpS1-60C antibody (1:2000) for 2 h, followed by alkaline phosphatase-conjugated anti-rat secondary antibody (Sigma); detection used BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) and NBT (Nitroblue tetrazolium chloride).

Immunolocalization

Papaver rhoeas pollen from plants with haplotypes S_1S_3 was grown in germination medium (GM)36 for 1 h at 25°C and prepared according to37, followed by incubation with the PrpS1-60C antibody (1:500 in TBS+ 1% BSA; 4°C overnight), then FITC-conjugated goat anti-rat antibody (1:50, 1.5 h). Cells were mounted on slides and examined by confocal microscopy (Bio-Rad Radiance 2000 MP) using single scans (100x plan-Apo 1.4 NA oil objective, Nikon). When pre-immune antiserum was used, no signal was obtained using identical additions and settings.

The *in vitro* bioassay for *Papaver* SI: use of this as an assay for function

We have used *in vitro* bioassays for demonstration of PrpS function instead of the alternative strategy of stable transformation with a *PrpS* allele because the latter is not

possible in *Papaver*. These bioassays have been routinely used to demonstrate *S*-specific function. Because it has not been possible to generate plants that are homozygous for *S* haplotypes in *Papaver*, we use full-sib families that are segregating for plants that are heterozygous for *S* haplotypes. This makes the interpretation of the data slightly more complicated, as we always have a mixed population of pollen grains in our samples.

The *Papaver* SI *in vitro* bioassay was initially used for demonstrating that stigmatic extracts had *S*-specific biological activity; we showed the distribution of individual pollen tube lengths for control, fully compatible, incompatible, and half-compatible interactions, with bimodal distribution of inhibition in a half-compatible SI response, as expected38. This SI bioassay was used to provide evidence for the pistil *S* gene (now named *PrsS*) being the *S* determinant39; we demonstrated *S*-allele-specific inhibition of pollen by recombinant $PrsS_1$ protein. This half-compatible interaction gave 40-44% mean inhibition (compared to the theoretical maximum of 50%) with pollen from plants with haplotypes S_1S_3 or S_1S_6, but no inhibition with pollen from plants with haplotypes S_3S_6 or S_2S_4. As we now have several cloned pistil *PrsS* alleles, for any *in vitro* SI bioassay, we routinely add two recombinant PrsS proteins to obtain a full SI response where all pollen is inhibited (see e.g.40-42).

For the SI *in vitro* bioassays carried out here, we used two recombinant PrsS proteins ($PrsS_1$ and $PrsS_3$ to pollen from plants with haplotypes S_1S_3, and $PrsS_3$ and $PrsS_8$ proteins to pollen from plants with haplotypes S_3S_8) to achieve a full SI response (all pollen inhibited). With the peptide bioassay (see below), we added the $PrpS_1$ peptide to the SI bioassay, so the expectation was that a maximum of 50% of pollen would be rescued if we obtained *S* haplotype-specific alleviation of SI. For the antisense oligonucleotide experiments we used pollen from plants with S_1S_3 and S_3S_8 haplotypes in combination with antisense-$PrpS_1$ and/or antisense-$PrpS_8$. The expectation was that *S*-haplotype-specific alleviation of SI would be obtained using antisense-$PrpS_1$ with pollen from plants with S_1S_3 haplotypes, but this would only be 50% rescue (pollen carrying $PrpS_1$, but not pollen carrying $PrpS_3$); we would also expect no alleviation of SI with antisense-$PrpS_1$ with the pollen from S_3S_8 plants. We also carried out experiments with antisense-$PrpS_8$ in these combinations, with the prediction that antisense-$PrpS_8$ will alleviate the SI response by a maximum of 50% with pollen from plants with S_3S_8 haplotypes (rescue of pollen carrying $PrpS_8$, but not $PrpS_3$). SI Fig 3 shows data for populations of individual pollen tubes for an experiment using a $PrpS_1$ antisense oligonucleotide added to an SI assay using pollen from plants with S_1S_3 or S_3S_8 haplotypes. The expectation is that $PrpS_1$ antisense oligonucleotide should rescue pollen carrying the $PrpS_1$ allele and not the $PrpS_3$ or $PrpS_8$ alleles. The plot shows that the rescue is clearly bimodal, with some pollen tubes still strongly inhibited, whilst others have alleviated inhibition, which gives confidence to the mean data shown in Fig 3.

We designed these experiments to be reciprocal so that we could demonstrate allelic specificity of rescue and to rule out some non-specific effects, as if the antisense-$PrpS_1$ caused pollen tubes to grow longer for some other reason, we should see this effect with the pollen from plants with S_3S_8 haplotypes. Sense oligonucleotides were used as additional controls, in order to demonstrate that the antisense oligonucleotides specifically had this effect on pollen tube growth. These assays provide a robust test of whether $PrpS_1$ and $PrpS_8$ are allelic, as we show that they have the same biological function and they exhibit allelic specificity. If $PrpS_8$ was not allelic to $PrpS_1$, then it would not affect pollen tube growth as predicted. Statistical tests were carried out using MINITAB. Tests comprised two-way comparisons between pairs of data using a student's t-test.

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Peptide bioassay

A 15 amino acid peptide corresponding to part of the predicted 35 amino acid $PrpS_1$ external loop region was designed and two randomized versions of this peptide were synthesized (see **Methods Summary**-peptide binding assay for details). *P. rhoeas* pollen was grown *in vitro* and SI induced using recombinant $PrsS_1$, $PrsS_3$ and $PrsS_8$12. SI-induced pollen inhibition was compared with SI in the presence of the $PrpS_1$ peptide or a randomized peptide at 200 $\mu g\ ml^{-1}$. Pollen grains and tubes were scored after 1h, using two categories: "inhibition" or "growth"; a minimum of 100 pollen grains/tubes was scored for each sample. Data were analysed using Fisher's Exact Test for 2×2 contingency tables43.

Primer details

Primers used for the isolation of *PrpS* alleles

Gene specific primers used to amplify full length *$PrpS_1$*

PRPS1-5′-UTR1 GTAGCATTTACAATCTTCTTAGAAATGC

PRPS1-3′-UTR1 GAGAACACGTCATTGGAATTATTGAG

5′ RACE primer to obtain another S allele (resulting in isolation of $PrpS_8$)

NH3′-3 GCGACCGAAGTGGCATG

Gene specific primers used to amplify *$PrpS_8$*

PRPS8 5′-1 GGCAGTTATGCCTCGACATGCAATTG

PRPS8 3′-1 TTAAACCTCAACACTACGGTGG

5′ RACE primer used to obtain full length $PrpS_8$

PRPS8 5′RACE-1 GCTGTGCAATCCTCTCTGATCAAG

Degenerate primers used to obtain *$PrpS_3$*

PRPS 5′-1 ATGCCACGAMRTGSAAKTGTTG

PRPS 5′-2 CCTATTKGGAKYCKCASTTGCC

PRPS 5′-3 GTAGTMGCATTTGGGACYRCTGC

PRPS 3′-1 GTGAACTTAGCAACAAYWGCRAGG

PRPS 3′-2 TCAAGTWKACTAGTARRAGCTTGCC

PRPS 3′-3 CCAAYYAAAAATCCYTCRGTCATGCC

3′ and 5′ RACE primers used to obtain full length $PrpS_3$

BGPRPS3-3′R1 GCTTCTACTGATTAACTTGTTCTCCGG

BGPRPS3-5′R1 CTGCAGTTGCATGCATATTGTGTGTCG

Gene-specific primers used for *S*-locus linkage analysis

Primers for pistil *$PrsS_1$*, *$PrsS_3$* and *$PrsS_8$*

SS1-5′ GCTATCGTTCTTCTTGTCCAAGTCAAGCGG

SS1-3′ CATCCCTCTTTGCCTGATAGGAATAAAACCCG

SS3-5′ GACTTTGGTTAGCATGTCCAATTCCATCGGC

SS3-3′ GTCCCTCTTGCCCGAGTAAGCATCG

SS8-5′ GTCCTTCTTGACCTTGGCCTCATCTCG

SS8-3′ CCGTGATCATCTCGTTGTGCTCGATAGG

Primers for *PrpS*$_1$, *PrpS*$_3$ and *PrpS*$_8$

PRPS1-5′ CAGTTTGTTGGAGGATTATGTACCCCGTTGG

PRPS1-3′ GCGACCACACAGATCATTATGGAAGATAAGAAGG

PRPS3-5′ GAGTAGCATTTCTCGTGAGAATTAAATCTCACCATGC

PRPS3-3′ GGTCACGACCCCAACCAATTGCAACG

PRPS8-5′ CGCACTTGCCATAAGAACTGTCATTTCTCACC

PRPS8-3′ CACTACGATCACGGTCCCAACCAGC

References for Online Methods

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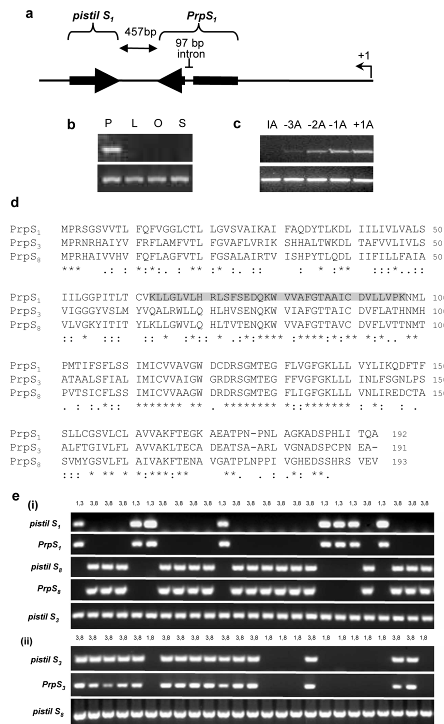

Fig 1. Organization and expression of *PrpS1*

(a) Organization of the S_1 locus. Arrows indicate pistil *PrsS1* and pollen *PrpS1* coding sequences and their orientation; transcription start site (+1). An intron is located 84 bp from the 3′ end.

(b) RT-PCR shows *PrpS1* is expressed in pollen (P) but not in leaf (L), ovary (O) or stigma (S) (*top panel*). (c) RT-PCR showing *PrpS1* expression increases during anther development. Immature anthers (IA); anthers 3 (-3A), 2 (-2A), 1 (-1A) days pre-anthesis, and at anthesis (+1A). Glyceraldehyde-3-phosphate dehydrogenase (GAPD) shows equal loading (*bottom panels*).

(d) Alignment of *PrpS1*, *PrpS3* and *PrpS8* deduced amino acid sequences. The predicted extracellular loop segment (TMHMM) is indicated for $PrpS_1$ (grey box).

(e) Linkage of *PrpS* and *PrsS* to the *S* locus. Full-sib families segregating for haplotypes (i) S_1S_3 and S_3S_8 and (ii) S_1S_8 and S_3S_8 were used for PCR. Pistil *PrsS1*-, *PrsS3*- and *PrsS8*- and pollen *PrpS1*, *PrpS3* and *PrpS8* sequences were amplified only if plants carried the corresponding *PrpS* allele. *S* haplotypes are indicated: S_1S_3 (1,3), S_3S_8 (3,8), S_1S_8 (1,8).

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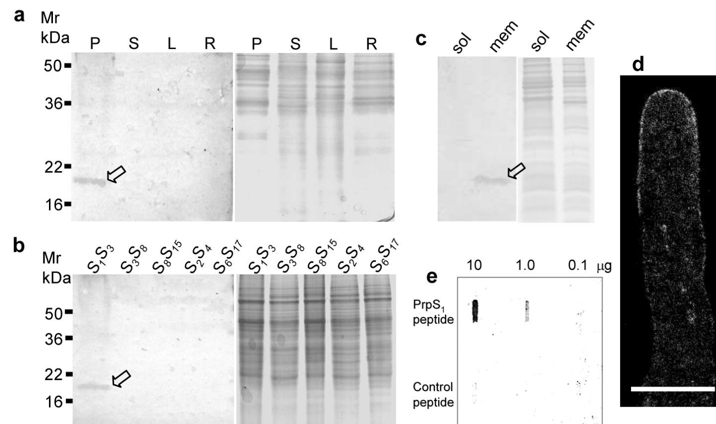

Fig 2. PrpS is pollen membrane-associated

(a) Western blotting detects $PrpS_1$ at ~20 kDa (arrow) in pollen (P), but not stigma (S), leaf (L) or root (R) membrane-enriched protein extracts (*left-hand panel*). Coomassie staining shows equal loading (*right-hand panel*).

(b) *$PrpS_1$* is expressed in pollen samples carrying *S_1*, but not other alleles (*left-hand panel*). Coomassie shows equal loading (*right-hand panel*).

(c) Western blot of fractionated pollen extracts. PrpS is not present in cytosolic extracts, but is present in a Triton-X-100 enriched fraction.

(d) Immunolocalization shows $PrpS_1$ localization to the pollen tube plasma membrane. Scale bar, 10 μm.

(e) $PrpS_1$ binds $PrsS_1$. A 15-mer peptide corresponding to part of the $PrpS_1$ predicted 35 amino acid extracellular loop region (DQKWVVAFGTAAICD) binds recombinant $PrsS_1$ in a concentration-dependent manner (*top panel*). A corresponding randomized peptide (FTVDVKDCAAAWGQI) did not bind $PrsS_1$ (*bottom panel*). Concentrations are as indicated; see **Methods** for details; n=8.

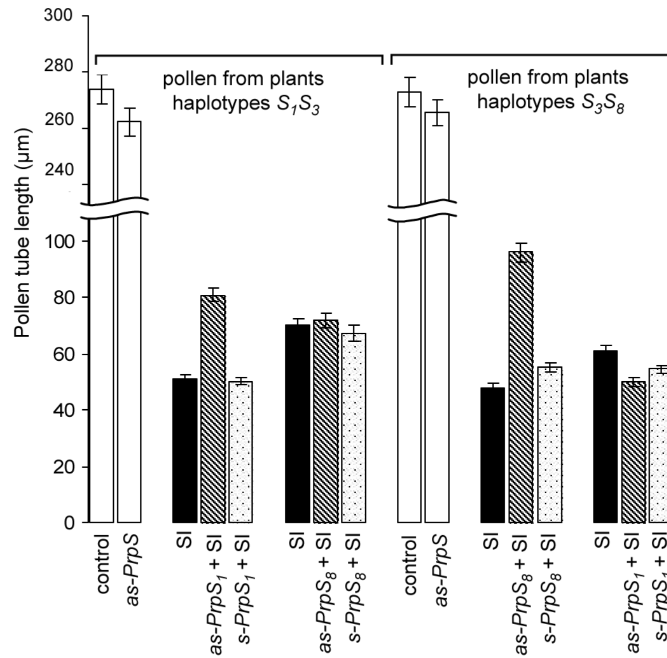

Fig 3. PrpS determines *S*-specific pollen inhibition

PrpS$_1$ and *PrpS*$_8$ antisense oligonucleotides (as-ODNs: *as-PrpS*$_1$, *as-PrpS*$_8$) "rescue" pollen from plants with S_1S_3 or S_3S_8 haplotypes, from SI-induced inhibition in an *S*-specific manner, while *PrpS*$_1$ and *PrpS*$_8$ sense oligonucleotides (s-ODNs: *s-PrpS*$_1$, *s-PrpS*$_8$) do not. Controls: untreated pollen and as-ODNs without SI induction controls (white bars); SI-induced pollen (black bars); SI-induced in presence of *as*-ODN (crosshatched bars); SI-induced in presence of s-ODNs (dotted bars). 50 pollen tubes were measured in three independent experiments (150 in total); error bars indicate s.e.m.

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