LETTERS

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

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Higher plants produce seed through pollination, using specific interactions between pollen and pistil. Self-incompatibility is an important mechanism used in many species to prevent inbreeding; it is controlled by a multi-allelic *S* locus[1,2]. '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 death[3–7]. Here we have cloned three alleles of a highly polymorphic pollen-expressed gene, *PrpS* (*Papaver rhoeas* pollen *S*), 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 (currently called *S*, but referred to hereafter as *PrsS* for *Papaver rhoeas* stigma *S* determinant). Sequence analysis indicates that *PrsS* and *PrpS* are equally ancient and probably 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* self-incompatibility 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.

It has been established that self-incompatibility has evolved independently several times. Three self-incompatibility systems have been well characterized at a molecular level[1,2]. 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 self-incompatibility system. Other characteristics expected of them are high levels of allelic polymorphism and tissue-specific expression. Most importantly, they should function in mediating the self-incompatibility response. To understand fully how different self-incompatibility 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 inhibition[3–7,11]. Although we identified a glycoprotein in pollen that bound to the pistil S protein, studies indicated that it was not the pollen *S* determinant, although it might modulate the self-incompatibility response[12]. Recent analysis of the S_1 locus enabled identification of the pollen component of the *S* locus on a cosmid clone comprising a 42-kilobase (kb) region at the S_1 locus.

Nucleotide sequencing and analysis identified a novel putative open reading frame (ORF) 457 base pairs (bp) from the S_1 pistil gene (Fig. 1a). Expression analysis using polymerase chain reaction with reverse transcription (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* gene[8]. These data suggested that this ORF was a candidate for the *Papaver* pollen *S* gene (designated *PrpS* for *Papaver rhoeas* pollen *S*). We propose renaming the gene that determines self-incompatibility specificity in pistils (currently designated as *S*) to provide a clearer nomenclature; we suggest *PrsS* (*Papaver rhoeas* stigma *S* determinant).

The cDNA of $PrpS_1$ comprises 1,206 bp containing a coding region of 579 bp encoding a 192-amino-acid polypeptide with a predicted molecular mass 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 molecular mass of 21.1 kDa (pI 6.57) and 20.9 kDa (pI 8.51), respectively. Southern blotting revealed that *PrpS* is a single-copy gene (Supplementary 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-sibling families segregating for these haplotypes (45 and 25 individuals, a total of 140 *PrpS*/*PrsS* pairs). $PrpS_1$ was amplified only from plants carrying the S_1 haplotype; $PrpS_3$ was amplified only from plants carrying the S_3 haplotype; 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 markedly 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 species[13–16]. *Papaver* is no exception, with the pistil proteins $PrsS_1$ and $PrsS_3$ showing 46% sequence divergence, $PrsS_1$ and $PrsS_8$ showing 40% divergence, and $PrsS_3$ and $PrsS_8$ with 46% divergence. PrpS proteins have a similar

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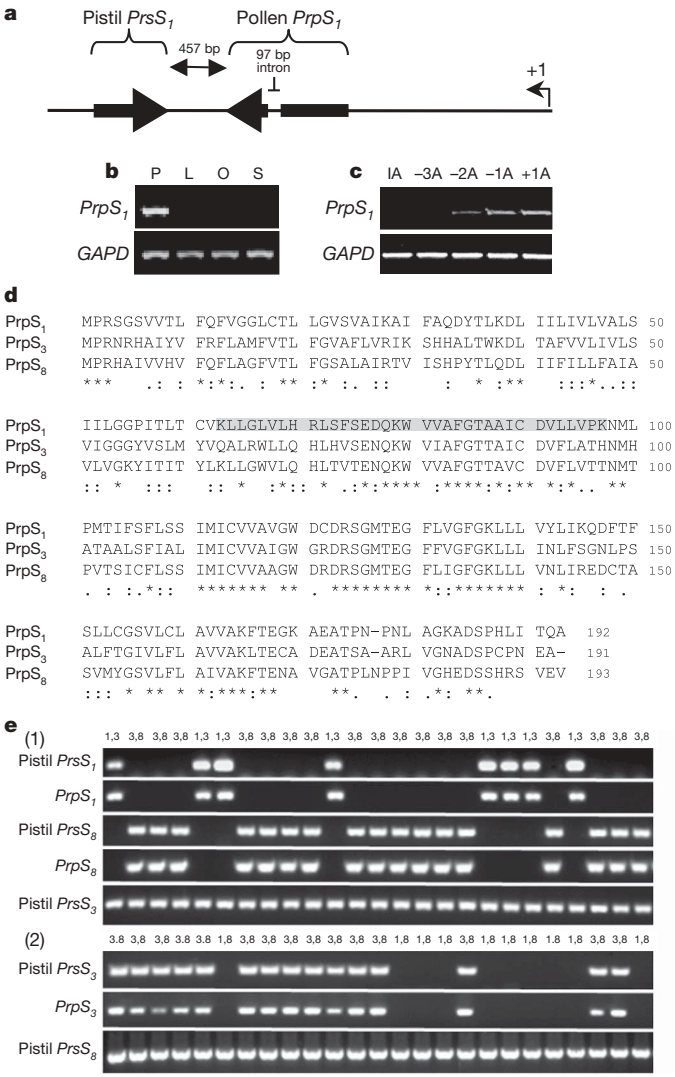

Figure 1 | Organization and expression of *PrpS*$_1$. **a**, Organization of the S_1 locus. Arrows indicate pistil *PrsS*$_1$ and pollen *PrpS*$_1$ coding sequences and their orientation. Transcription start site (+1) is shown. An intron is located 84 bp from the 3′ end. **b**, RT–PCR shows that *PrpS*$_1$ is expressed in pollen (P) but not in leaf (L), ovary (O) or stigma (S). **c**, RT–PCR showing that *PrpS*$_1$ expression increases during anther development. IA, immature anthers; −3A, −2A, −1A, anthers 3, 2 and 1 days pre-anthesis, respectively; +1A, anthesis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) shows equal loading. **d**, Alignment of PrpS$_1$, PrpS$_3$ and PrpS$_8$ deduced amino acid sequences. The predicted extracellular loop segment (TMHMM) is indicated for PrpS$_1$ (grey box). '*', identical; ':', conserved, '.', semi-conserved amino acid substitutions. **e**, Linkage of *PrpS* and *PrsS* to the *S* locus. Full-sibling families segregating for haplotypes (1) S_1S_3 and S_3S_8 and (2) S_1S_8 and S_3S_8 were used for PCR. Pistil *PrsS*$_1$, *PrsS*$_3$ and *PrsS*$_8$, and pollen *PrpS*$_1$, *PrpS*$_3$ and *PrpS*$_8$ 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).

level of polymorphism (Fig. 1d): the PrpS$_1$ and PrpS$_3$ predicted amino acid sequences are 50% divergent; PrpS$_1$ and PrpS$_8$ show 40% divergence; and PrpS$_3$ and PrpS$_8$ are 47% divergent.

The pollen and pistil *S* determinants should show evidence of co-evolution[16]. Examination of the *PrpS* sequences for non-synonymous to synonymous (K_a/K_s) substitutions reveal that the *PrsS* alleles have a mean K_a/K_s ratio of 0.234, and the *PrpS* alleles have a mean K_a/K_s ratio of 0.368 (Supplementary Table 1). A two-tailed *t*-test showed no significant difference between substitution rates in *PrpS* and *PrsS* genes. These data indicate 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 programs, 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 and Supplementary Fig. 2). In support of predictions that PrpS is a transmembrane protein, western blot 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; see also Supplementary 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, whereas the corresponding randomized peptide did not (Fig. 2e).

To determine whether PrpS is functionally involved in the self-incompatibility response, we investigated whether it mediates *S*-specific pollen inhibition, using *in vitro* self-incompatibility bioassays[8]. Peptides based on extracellular domains of receptors have been used to identify ligand-binding epitopes via their ability to block the receptor–ligand interaction[18]. Preliminary experiments with the peptide used in the binding assay tested whether it could block self-incompatibility-mediated inhibition. Pollen from plants with haplotypes S_1S_3, when challenged with incompatible recombinant PrsS, were rescued from inhibition by PrpS$_1$ peptides ($n = 6$; $P < 0.001$; Supplementary Fig. 3 and Supplementary Table 2) whereas randomized peptides based on the same amino acids had no effect ($n = 6$; not significant, Supplementary Fig. 3 and Supplementary Table 2). 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 approach[3,19]. 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 self-incompatibility *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

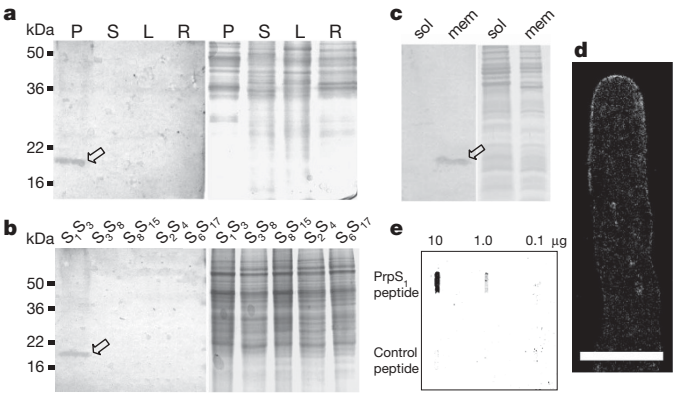

Figure 2 | PrpS is pollen-membrane associated. **a**, Western blot detects PrpS$_1$ at ~20 kDa (arrow) in pollen (P), but not in stigma (S), leaf (L) or root (R) membrane-enriched protein extracts (left panel). Coomassie staining shows equal loading (right panel). **b**, *PrpS*$_1$ is expressed in pollen samples carrying S_1, but not other alleles (left panel). Coomassie staining shows equal loading (right panel). **c**, Western blot of fractionated pollen extracts. PrpS is not present in cytosolic extracts (sol), but is present in a Triton-X-100 enriched fraction (mem). **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). A corresponding randomized peptide (FTVDVKDCAAAWGQI) did not bind PrsS$_1$ (bottom). Concentrations are as indicated; see Methods for details; $n = 8$.

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 50% of pollen (carrying S_1).

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

To confirm further the *S*-specific effect of the as-ODNs, we also tested their effect on pollen from plants with haplotypes S_3S_8. Self-incompatibility resulted in inhibited pollen tubes (19.8% of the control, $n = 300$), and addition of $PrpS_8$ as-ODNs alleviated the self-incompatibility-induced inhibition, giving a highly significant 100.3% increase in pollen tube length ($P < 0.001$, $n = 150$), whereas there was no effect using $PrpS_1$ as-ODNs ($P = 0.336$, not significant, $n = 150$) or $PrpS_8$ s-ODNs ($P = 0.565$, not significant, $n = 150$) (Fig. 3). Together these data demonstrate that PrpS has a crucial role in self-incompatibility-induced *S*-haplotype-specific pollen tube inhibition.

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. Self-incompatibility in *Papaver* is distinct from the other well characterized self-incompatibility systems (that is, from both the *Brassica* pollen *S* determinant SCR/SP11 and the pollen F-box protein SLF/SFB from the S-RNase-based self-incompatibility system)[1,20–23]. As *PrpS* has no homologues, its nature is intriguing. Self/non-self discrimination and other recognition systems which are controlled by a highly polymorphic locus are not limited to self-incompatibility; 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/non-self recognition systems.

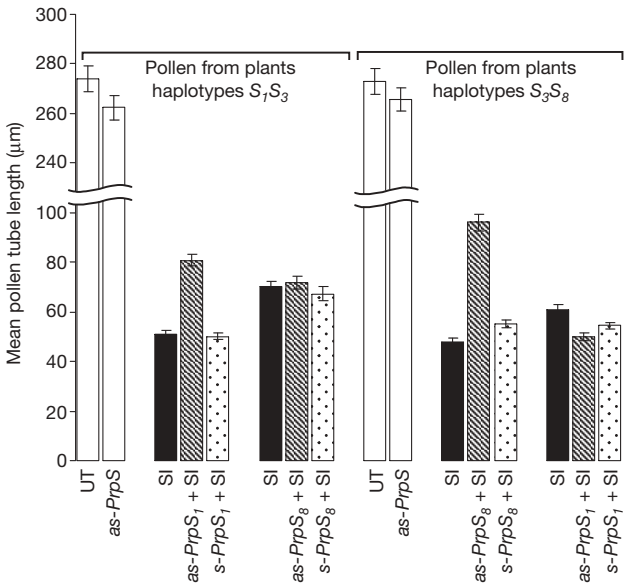

Figure 3 | PrpS determines *S*-specific pollen inhibition. $PrpS_1$ and $PrpS_8$ antisense oligonucleotides (as-ODNs: *as-PrpS*$_1$, *as-PrpS*$_8$) 'rescue' pollen from plants with S_1S_3 or S_3S_8 haplotypes from self-incompatibility (SI)-induced inhibition in an *S*-specific manner, whereas $PrpS_1$ and $PrpS_8$ sense oligonucleotides (s-ODNs: *s-PrpS*$_1$, *s-PrpS*$_8$) do not. Controls: untreated (UT) pollen and as-ODNs without self-incompatibility induction (white bars); self-incompatibility-induced pollen (black bars); self-incompatibility-induced in the presence of as-ODN (crosshatched bars); self-incompatibility-induced in the presence of s-ODNs (dotted bars). Fifty pollen tubes were measured in three independent experiments (150 in total); error bars indicate s.e.m.

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 *P. 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 analysed using BLAST (http://ncbi.nlm.nih.gov/BLAST) and ORF Finder (http://searchlauncher.bcm.tmc.edu)[28]. The organization 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 Methods for primer details) on pollen cDNA from suitable *S*-haplotypes, using low annealing temperatures (48 °C).

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

Peptide binding assay. A 15-amino-acid peptide (DQKWVVAFGTAAICD) corresponding to part of the predicted extracellular loop segment of $PrpS_1$ (TMHMM; http://www.cbs.dtu.dk/services/TMHMM30) and a randomized version (FTVDVKDCAAAWGQI) were synthesized (Alta Bioscience). 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 anti-$PrsS_1$ antisera and alkaline phosphatase detection.

Antisense oligonucleotide 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, aattGCTGTGCTGGTGggaa; lowercase 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 before induction of self-incompatibility with recombinant $PrsS_1$, $PrsS_3$ and $PrsS_8$ (ref. 8). After 2 h, pollen tubes were fixed in 2% formaldehyde and 150 pollen tube lengths were measured in three independent experiments.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.J.W., B.H.J.d.G. and N.H. contributed equally to this work. F.C.H.F. and V.E.F.-T. are joint senior authors.

Author Information *PrpS*$_1$, *PrpS*$_3$ and *PrpS*$_8$ sequences have been deposited in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) as accessions AM743176, FN178511 and AM743177. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to V.E.F.-T. (V.E.Franklin-Tong@bham.ac.uk).

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 EcoRV, BamHI, Pst1 or BglII were probed with a $PrpS_1$ probe. Blots were hybridized 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 programs: TMHMM2.0[32], PredictProtein, SOSUI, HMMTOP, TMpred, TM-Finder, SPLIT 4, ConPredII, Phobius. TMHMM2.0 in particular is regarded as a very robust transmembrane helix predictor, although predicting the number of membrane-spanning regions remains difficult[33]. 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 three or five transmembrane 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 three and five transmembrane proteins are rare (A. Lovering, personal communication). All the predictions indicate an extracellular loop segment. Supplementary Fig. 2 shows a cartoon indicating predictions for the regions; this is a tentative assignment of possible topology.

Linkage analysis. Segregation analysis was carried out on individual plants from several full-sibling 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 analysed for *S* allele segregation for at least seven generations in a pedigree going back to 1994.

The number of plants required to be analysed, in order to be statistically confident at 95% rejection level[34] that there is no recombination, is 25 plants (one cannot formally provide evidence that there is never recombination). We carried out analysis of a full-sibling 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, and analysed 45 full-sibling 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 $PrsS_1$, $PrsS_3$ and $PrsS_8$, and $PrpS_1$, $PrpS_3$ and $PrpS_8$ sequences. (See Supplementary 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 synthesized (Omniscript RT kit, QIAGEN). Gene-specific primers (see Supplementary 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 carboxy terminus of $PrpS_1$ (designated PrpS1-60C) was expressed as a His-tagged recombinant protein using pET21b (Novagen). Recombinant protein was isolated from *Escherichia coli* BL21 (DE3) using Ni-NTA resin following the manufacturer's (QIAGEN) protocol. Antisera (PrpS1-60C) was raised in rats (ISL Immune Systems).

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 determined[35] 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 (1:2,000) 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 to the method of ref. 37, 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 (×100 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* self-incompatibility: 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-sibling 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* self-incompatibility *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 self-incompatibility response, as expected[38]. This self-incompatibility bioassay was used to provide evidence for the pistil *S* gene (for which we propose the name *PrsS*) being the *S* determinant[39]; 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* self-incompatibility bioassay, we routinely add two recombinant PrsS proteins to obtain a full self-incompatibility response where all pollen is inhibited (see for example refs 40–42).

For the self-incompatibility *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 self-incompatibility response (all pollen inhibited). With the peptide bioassay (see below) we added the $PrpS_1$ peptide to the self-incompatibility bioassay, so the expectation was that a maximum of 50% of pollen would be rescued if we obtained *S*-haplotype-specific alleviation of self-incompatibility. 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 self-incompatibility would be obtained using antisense $PrpS_1$ with pollen from plants with S_1S_3 haplotypes, but this would only rescue 50% (pollen carrying $PrpS_1$, but not pollen carrying $PrpS_3$); we would also expect no alleviation of self-incompatibility 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 self-incompatibility response by a maximum of 50% with pollen from plants with S_3S_8 haplotypes (rescue of pollen carrying $PrpS_8$, but not $PrpS_3$). Supplementary Fig. 3 shows data for populations of individual pollen tubes for an experiment using a $PrpS_1$ antisense oligonucleotide added to a self-incompatibility 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, whereas 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 nonspecific 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.

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 for details). *P. rhoeas* pollen was grown *in vitro* and self-incompatibility induced using recombinant $PrsS_1$, $PrsS_3$ and $PrsS_8$ (ref. 42). Self-incompatibility-induced pollen inhibition was compared with self-incompatibility in the presence of the $PrpS_1$ peptide or a randomized peptide at 200 μg ml^{-1}. Pollen grains and tubes were scored after 1 h, 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 tables[43].

Primers used for the isolation of *PrpS* alleles. Gene-specific primers used to amplify full-length $PrpS_1$: PRPS1-5′-UTR1 GTAGCATTTACAATCTTCTTA GAAATGC; 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 GGCAGTTATG CCTCGACATGCAATTG; 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 ATGCCACGAMR TGSAAKTGTTG; PRPS 5′-2 CCTATTKGGAKYCKCASTTGCC; PRPS 5′-3 GT AGTMGCATTTGGGACYRCTGC; PRPS 3′-1 GTGAACTTAGCAACAA YWGCRAGG; 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 CTGCAGTTGC ATGCATATTGTGTGTCG.

Gene-specific primers used for *S*-locus linkage analysis. Primers for pistil $PrsS_1$, $PrsS_3$ and $PrsS_8$: SS1-5′ GCTATCGTTCTTCTTGTCCAAGTCAAGC GG; SS1-3′ CATCCCTCTTTGCCTGATAGGAATAAAACCCG; SS3-5′ GACTT TGGTTAGCATGTCCAATTCCATCGGC; SS3-3′ GTCCCTCTTGCCCGAGT AAGCATCG; SS8-5′ GTCCTTCTTGACCTTGGCCTCATCTCG; SS8-3′ CCGT GATCATCTCGTTGTGCTCGATAGG.

Primers for $PrpS_1$, $PrpS_3$ and $PrpS_8$: PRPS1-5′ CAGTTTGTTGGAGGATT ATGTACCCCGTTGG; PRPS1-3′ GCGACCACACAGATCATTATGGAAGAT AAGAAGG; PRPS3-5′ GAGTAGCATTTCTCGTGAGAATTAAATCTCACCA TGC; PRPS3-3′ GGTCACGACCCCAACCAATTGCAACG; PRPS8-5′ CGCAC TTGCCATAAGAACTGTCATTTCTCACC; PRPS8-3′ CACTACGATCACGGT CCCAACCAGC.

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CORRIGENDUM

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Corrigendum: Identification of the pollen self–incompatibility determinant in *Papaver rhoeas*

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Recently, it has come to our attention that in the left panel of Fig. 2b of this Letter, the lanes labelled S_2S_4 and S_6S_{17} were duplicated. We have reviewed the original data. It seems likely that a duplicated part of the blot was placed over lane S_6S_{17} to aid alignment of molecular mass markers and inadvertently left there. We have now removed the duplicated lane and show the whole western blot (Fig. 1). Our conclusions are unaffected.

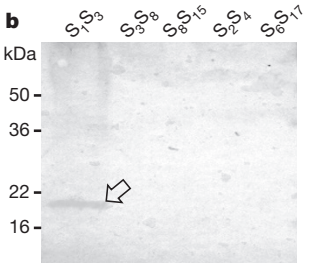

Figure 1 | This is the corrected left panel of Fig. 2b. The right panel showing the Coomassie staining is not shown.