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Title: The *Papaver rhoeas S*-determinants confer self-incompatibility to *Arabidopsis thaliana in planta*

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Abstract:

Self-incompatibility (SI) is a major genetically controlled system used to prevent inbreeding in higher plants. *S*-determinants regulate allele-specific rejection of "self" pollen by the pistil. SI is an important model system for cell-cell recognition/signaling and could be potentially useful for F_1 hybrid breeding. To date, transfer of *S*-determinants has utilized complementation of orthologs to "restore" SI in close relatives. We expressed the *Papaver rhoeas S*-determinants, *PrsS* and *PrpS*, in *Arabidopsis thaliana*. This enabled pistils to reject pollen expressing cognate *PrpS*. Moreover, plants co-expressing cognate *PrpS* and *PrsS* exhibit robust SI. This demonstrates that PrsS and PrpS are sufficient for a functional synthetic *S*-locus *in vivo*. This represents the first transfer of novel *S*-determinants into a highly divergent species (>140 *m.y.* apart) with no orthologs.

One Sentence Summary:

The *Papaver rhoeas S*-determinants *PrsS* and *PrpS* confer self-incompatibility to *Arabidopsis thaliana in planta*

Main Text:

Many plants are hermaphrodites, with male and female organs in close proximity. As this risks self-fertilization and undesirable inbreeding depression, many plants utilize self-incompatibility (SI) as a mechanism to prevent selfing. SI is controlled by a *S*-locus allowing self/non-self

recognition between pistil and pollen (*1*, *2*). SI in *Papaver rhoeas* is gametophytically controlled and specified by a pistil *S*-determinant, *PrsS* (*P. rhoeas stigma S* (*3*)) and a pollen *S*-determinant, *PrpS* (*P. rhoeas pollen S* (*4*)). PrsS and PrpS interact to trigger a signaling network in incompatible pollen, resulting in Programmed Cell Death (PCD) (*5-8*). *Arabidopsis thaliana* is a self-fertile member of the Brassicaceae. Self-compatibility in Arabidopsis originated recently (<0.5 *mya*) (*9*), through loss/inactivation of their *S*-determinants, SRK (*10*) and SCR (*11*). We recently demonstrated that pollen from *A. thaliana* expressing *PrpS-GFP* was inhibited by cognate recombinant PrsS proteins and displayed hallmark features of *Papaver* SI (*8*). Here we have expressed PrsS in *A. thaliana* pistils and show that they reject cognate pollen. Moreover, *A. thaliana* pistils co-expressing *PrsS* and *PrpS* set no self-seed. This demonstrates that *PrsS* and *PrpS* function as *S*-determinants and are the sole additional requirement to elicit SI. Intergeneric transfer of *S*-determinants has only been achieved between closely related species with orthologs of the *Brassica S*-determinants, effectively involving complementation using *SRK* and *SCR* pairs to "restore" SI (*12-14*). Because the Papaveraceae diverged from the Brassicaceae ~140 *mya* (*8*, *15*) and Arabidopsis lacks *PrsS* and *PrpS* orthologs, finding that they function *in planta* in *A. thaliana* is a milestone.

PrsS encodes a small secreted protein, specifically and developmentally expressed in *P.rhoeas* stigmas (*3*). The promoter of *S Locus-Related 1 (SLR1)* gene from *Brassica oleracea* directs stigma-specific, developmentally-regulated gene expression (*16*, *17*), and exhibits maximal expression at flower maturity (*16*). Here we show that expression of *SLR1p* in Arabidopsis (**Fig 1A, Fig S1**) is spatiotemporally indistinguishable from SRK in *B. oleracea* (*16*). Therefore, we used *SLR1p* to drive expression of $PrsS_1$ in Arabidopsis by transforming a *SLR1p::*$PrsS_1$

construct into *Col-0* (*At-PrsS$_1$* lines). RT-PCR analysis of pistil mRNA from 10 independent lines revealed differing *PrsS$_1$* transcript levels (**Fig 1B**). Western analysis of pistil extracts confirmed the presence of PrsS$_1$ (**Fig 1C**). We also transformed *Col-0* with a *SLR1p::PrsS$_3$* construct to make *At-PrsS$_3$* lines. RT-PCR analysis revealed similar transgene expression to the highest-expressing *At-PrsS$_1$* line (**Fig 1D**).

To test the functionality of the *At-PrsS$_1$* lines we performed *semi-vivo* pollination assays (**Fig S2**) on excised pistils from the *At-PrsS$_1$* lines, using pollen from an *A. thaliana* line expressing *PrpS$_1$-GFP* (*8*), referred to as *At-PrpS$_1$* hereafter, examining the ability of *At-PrsS$_1$* stigmas to inhibit *At-PrpS$_1$* ("incompatible") pollen tube growth.

At-PrsS$_1$ line 9 pistils inhibited *At-PrpS$_1$* pollen tubes more strongly than line 4, while *Col-0* pollen was not inhibited (**Fig 2A**). Quantitation revealed that in *At-PrsS$_1$* pistils, *At-PrpS$_1$* pollen tubes were significantly shorter than *Col-0* pollen tubes ($P<0.001$, *t-test*, n=40; **Fig 2B**). After 70 min on *At-PrsS$_1$* pistils, *At-PrpS$_1$* pollen tubes from eight out of ten lines were <300 µm; *Col-0* controls were >300 µm (n=40; **Fig 2B**). Thus, *At-PrsS$_1$* pistils support pollen tube growth, but reject *At-PrpS$_1$* pollen. At 110 min, *At-PrpS$_1$* pollen tubes in *At-PrsS$_1$* pistils remained shorter than controls (**Fig S3**). The level of inhibition of *At-PrpS$_1$* pollen tubes in *At-PrsS$_1$* pistils correlated with *PrsS$_1$* expression levels (**Fig S4**). This provides strong evidence that *PrsS$_1$* functions in *A. thaliana* pistils to inhibit *At-PrpS$_1$* pollen.

A key feature of SI is *S*-allele specific inhibition of pollen. To test this, we pollinated excised *At-PrsS$_1$* or *At-PrsS$_3$* pistils with *At-PrpS$_1$* pollen or pollen from a line expressing *PrpS$_3$-GFP* (*8*),

referred to as *At-PrpS*$_3$ hereafter (**Fig 2C**). Strong pollen tube inhibition was observed only with cognate combinations of *At-PrsS* with *At-PrpS* (**Fig 2C, i, v**). Pollinations using non-cognate combinations of *At-PrsS* with *At-PrpS* resulted in normal pollen tube growth (**Fig 2C, ii, iv**). Controls (**Fig 2C, iii, vi, vii, viii, ix**) had long pollen tubes. *In vivo* pollinations of *At-PrsS* pistils also revealed differential inhibition of pollen tubes after 18 h (**Fig S5**). This demonstrates *S*-specific pollen tube inhibition by *A. thaliana* expressing PrsS.

With SI, pollination between cognate pollen and pistil *S*-alleles results in no seed production. *In planta* pollinations on *At-PrsS*$_1$ and *At-PrsS*$_3$ stigmas using cognate (incompatible) *At-PrpS*$_1$ and *At-PrpS*$_3$ pollen gave dramatically reduced silique lengths (6.2 ±1.4 and 6.3 ±1.7 mm respectively) compared to *Col-0* controls ($p<0.001$ ***, *t-test*, n=10; **Fig 2D-E, Fig S6, Table 1**). In contrast, *At-PrsS*$_1$ and *At-PrsS*$_3$ pistils pollinated with non-cognate (compatible) pollen resulted in normal silique lengths, like *At-PrsS*$_1$ and *At-PrsS*$_3$ pistils pollinated with *Col-0* pollen ($p=0.397$, *ANOVA*, n=10), and pollination of *Col-0* stigmas with *At-PrpS*$_1$ or *At-PrpS*$_3$ pollen ($p=0.871$, *ANOVA*, n=10; **Table 1**) resulted in normal siliques, similar to selfed *Col-0* siliques, demonstrating *At-PrsS* stigma and *At-PrpS* pollen are functional.

Analyzing self-seed set, many siliques were completely empty (7/10 for *At-PrsS*$_1$ pollinated with *At-PrpS*$_1$ and 6/10 for *At-PrsS*$_3$ pollinated with *At-PrpS*$_3$); seed-set for cognate pollinations was between 0.5 ±1.0 and 1.2 ±1.8 seeds/silique (n=20; **Table 1)**. Pollinations between non-cognate combinations resulted in normal seed-set (50.6 ±5, 50.0 ±3.9, n=10), significantly different from those with cognate combinations ($p<0.001$, ***, *t-test*, n=10). Pollinations between *Col-0* pistils and *At-PrpS*$_1$ or *At-PrpS*$_3$ pollen gave normal seed-set (49.9 ±3.7 and 47.6 ±3.7, n=10), so

transgenic stigmas and pollen are fully functional. Thus, the *Papaver S*-determinants function *in vivo* in an *S*-specific manner, resulting in failure of fertilization with cognate, but not non-cognate, pollen expressing *PrpS*.

We generated *Col-0* lines co-expressing $PrsS_1$ and $PrpS_1$ (SI_1-lines) by transforming homozygous *At-*$PrpS_1$-GFP plants with *SLR1p::*$PrsS_1$. Lines co-expressing $PrsS_3$ and $PrpS_1$ (*SC*-lines) were also generated. Expression of $PrsS_1$ and $PrpS_1$ was examined in three SI_1-lines (**Fig 3A**). Fluorescence microscopy of pollen from these SI_1-lines confirmed the expression of $PrpS_1$-GFP (**Fig 3B**). The SI_1- and *SC*-lines had a similar vegetative phenotype to *Col-0*, *At-*$PrpS_1$ and *At-*$PrsS_1$ plants (**Fig S7**). However, when left to set self-seed naturally, the SI_1-line plants had small siliques (**Fig 3C, Fig S7F**), between 3 ±0.5 and 7 ±1.4 mm long (n=470; **Fig S8A**), significantly shorter than siliques of control plants (**Fig 3C, Fig S7F**) *Col-0* (15.5 ±0.6 mm), *At-*$PrpS_1$ (16.3 ±1.0), *At-*$PrsS_1$ (15.9 ±0.5) and *SC* plants (15.3 ±0.5 mm; $p<0.001$ ***, *t-test*; n=10 per plant). Twelve of the SI_1-lines set no seed; the remaining 35 plants had between 0.1 ±0.3 and 7.0 ±1.4 seeds/silique (n=350; **Fig S8B**). This was significantly less ($p<0.001$ ***) than the 58 ±1.6 seeds/silique in *Col-0* plants, *At-*$PrpS_1$ plants (57.7 ±2.8), *At-*$PrsS_1$ plants (58.3 ±1.6) and *SC*-lines (57.1 ±1.7; n=10). Total self seed-set from these SI_1-lines gave between 0 and 680 seeds; ~60% had <100 seeds per plant (**Fig S8C**). Self seed-set of control plants was >8,500 seeds/plant (n=12). This SI response is stronger than previously obtained using the *S*-determinants from *A. lyrata* (*12*, *18*) and similar to that achieved by (*19*). Lines co-expressing $PrsS_3$ and $PrpS_3$ (SI_3-lines) had a similar vegetative phenotype to *Col-0* plants except for short siliques (**Fig S9**). Self-seed-set analysis revealed small siliques (**Fig S10A, B**) and no/very low seed-set (**Fig S10B, C**), which were similar to those for the SI_1-lines. Analysis of naturally self-

pollinated pistils from *SI*-lines revealed that pollen tubes were inhibited in the upper pistil, while comparable self-pollinated *Col-0* pistils had pollen tubes extending through the pistil (**Fig 3E, F, Fig S11**, **Fig S12**). Together, these data provide compelling evidence that the *SI*-lines are self-incompatible.

To confirm that *SI*-lines were fully functional, pistils from representative SI_1-lines (SI_1*-9*, SI_1*-18* and SI_1*-32*) were pollinated with *At-*$PrpS_3$ or *Col-0* pollen (**Fig 3D**, n=9). Siliques obtained were not significantly different from those pollinated using *Col-0* stigmas (p=0.246, p=0.703, *ANOVA*; n=3). Pollen from SI_1-lines was also pollinated to *At-*$PrpS_1$ stigmas. They produced siliques and seed set not significantly different from *At-*$PrpS_1$ stigmas pollinated with *Col-0* pollen (p=0.931, p=0.803, *ANOVA*; n=3; **Fig S13A, B**). As pollen and pistils from these *SI*-lines are functional, the reason why these *SI*-lines set no self-seed is not because they have a fertility defect, but because they are self-incompatible.

In summary, our data provide compelling evidence that the *Papaver S*-determinants co-expressed in *A. thaliana* make plants self-incompatible and are the sole additional requirement to establish SI in this highly diverged self-compatible species. This is a milestone, as successful transfer of *S*-determinants to date has been between close relatives sharing an ancestral SI system, using complementation to "restore" SI (*12, 13*). Because the *Papaveraceae* and Brassicaceae are evolutionarily separated by ~140 million years (*8, 15*), our finding that they function *in planta* in *Col-0* to display a robust SI rejection response is of considerable interest. We are not "restoring" a SI system as SI in Brassica/Arabidopsis has genetically and functionally distinct *S*-determinants. As we previously showed that recombinant PrsS can trigger SI-PCD in

Arabidopsis pollen expressing PrpS (*8*) and there is no evidence that Brassica/Arabidopsis SI involves PCD, the most economical explanation is that the *Papaver S*-determinants can interface with, and activate, a network of common signaling components that mediate PCD to induce a "*Papaver*-like" SI response in Arabidopsis pollen. *Papaver* SI uses Ca^{2+}, reactive oxygen species and pH (*7, 20*), which have all been described in Arabidopsis signaling networks achieving various physiological responses, including PCD (*21*). We hypothesize that these common signaling components are co-opted downstream of PrsS-PrpS interaction to mediate SI. Our findings reinforce proposals that SI may recruit pre-existing signaling networks from other biological processes (*8, 22*). This raises questions about how SI systems evolved, as well as about recruitment and functional diversification of pre-existing components (*23*).

Wide transgenera functionality of the *Papaver* SI system opens up the possibility that transfer of these *S*-determinants may, in the longer-term, provide a tractable SI system for crop plants. Use of the *SLR1 promoter* from Brassica (*16, 17*) allows *PrsS* to be expressed in mature *Col-0* pistils unlike older *Col-0* pistils expressing *SCRb-SRKb* (*12, 18*). The production of F_1 hybrid plants in normally self-compatible species typically utilizes laborious, expensive manual emasculation to prevent self-fertilization. Transferal of a SI system into self-compatible species as an alternative method for the production of F_1 hybrids has been a long-term goal of SI research, with implications for solving Food Security issues.

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V.E.F-T. and F.C.H.F. are co-inventors on a patent application (2691/KOLNP/2011) filed by University of Birmingham relating to PrsS and PrpS. Materials will be freely available upon request for research purposes.

The authors declare that they do not have other competing financial interests.

The data reported here are available in Supplementary Materials.

Fig. 1. **Expression of the *SLR1* promoter is developmental-, tissue-specific and drives expression of *$PrsS_1$* in *A. thaliana*.**

(A) RT-PCR (*top*) shows *SLR1p::GFP* developmentally expressed in pistils and not expressed in stamen, petal, or leaf tissue . *GAPC* shows equal loading.

(B) RT-PCR of pistils from *At-$PrsS_1$* lines shows expression of *$PrsS_1$* (*top*) and quantitation of *$PrsS_1$* expression relative to *GAPC* (n=3, ± S.D., *below*).

(C) Western blot (α-$PrsS_1$ antisera) shows expression of $PrsS_1$ in *At-$PrsS_1$*.

(D) RT-PCR: expression of *$PrsS_3$* in *At-$PrsS_3$* line 8 is comparable to *At-$PrsS_1$* line 9.

Fig. 2. *At-PrpS* pollen is inhibited on cognate *At-PrsS* pistils, demonstrating *S*-specificity.

(A) Aniline blue staining of representative *semi-in-vivo* pollinations of *At-$PrsS_1$* pistils with *At-$PrpS_1$* or *Col-0* pollen.

(B) Quantitation of pollen tube lengths on *At-$PrsS_1$* pistils using *At-$PrpS_1$* pollen (*left*) or *Col-0* pollen (*right*); n= 4 stigmas/*At-$PrsS_1$* line.

(C) *At-$PrsS_1$* and *At-$PrsS_3$* pistils pollinated *semi-in-vivo* with *At-$PrpS_3$* or *At-$PrpS_1$* pollen. *At-PrpS* pollen tubes were inhibited on cognate *At-PrsS* pistils (i,v), while controls did not.

(D) Representative *in-vivo* pollination of an *At-$PrsS_1$* stigma with *At-$PrpS_1$* pollen resulted in a small, empty silique.

(E) *Col-0* pollinated with *Col-0* pollen had normal length silique and many seeds.

Fig 3. *A. thaliana* co-expressing *$PrsS_1$* and *$PrpS_1$* are self-incompatible and set no seed.

(A) RT-PCR of 3 *A. thaliana* *SI_1*-lines co-expressing *$PrsS_1$* and *$PrpS_1$*.

(B) Pollen from *SI_1*-lines exhibits GFP fluorescence (*top*); *Col-0* pollen has weak autofluorescence.

(C) Self-seed set: *SI_1*-lines formed short siliques; controls, including a *SC*-line co-expressing *$PrsS_3$* and *$PrpS_1$-GFP*, set normal siliques

(D) A selfed *SI_1*-plant gave small siliques; pollinations with *Col-0* or *At-$PrpS_3$* pollen gave normal siliques.

(E) Aniline blue staining of a self-pollinated *SI_1*-line pistil; pollen tubes are inhibited in the stigma/style.

(F) Self-pollinated *Col-0* pistil had long pollen tubes.

Table 1. *In vivo* pollination of *At-PrsS* stigmas with cognate *At-PrpS* pollen resulted in shorter siliques and no seed set.

Pollination of emasculated *At-PrsS$_1$* stigmas with *At-PrpS$_1$* pollen resulted in short siliques and reduced seed number, as did pollination of *At-PrsS$_3$* with *At-PrpS$_3$* pollen. Other control pollinations: non-cognate pollination of *At-PrsS$_1$* stigmas with *At-PrpS$_3$* pollen, *At-PrsS$_3$* stigmas with *At-PrpS$_1$* or *Col-0* pollen, *Col-0* stigmas with *Col-0*, *At-PrpS$_1$* or *At-PrpS$_3$* pollen gave normal silique length and seed number (mean ±S.D., n=10).

♀ \ ♂		*At-PrpS$_1$*	*At-PrpS$_3$*	*Col-0*
At-PrsS$_1$ (line 9)	Silique lengths (mm)	**6.2** ± **1.4**	16.1 ± 0.8	16.4 ± 0.7
	Seeds per silique	**0.5** ± **1.0**	50.6 ± 5.1	49.3 ± 5.3
At-PrsS$_3$ (line 8)	Silique lengths (mm)	16.4 ± 0.8	**6.3** ± **1.7**	16.5 ± 0.5
	Seeds per silique	50.0 ± 3.9	**1.2** ± **1.8**	50.0 ± 3.2
Col-0	Silique lengths (mm)	16.6 ± 1.0	16.6 ± 0.8	16.4 ± 0.7
	Seeds per silique	49.9 ± 3.7	47.6 ± 3.7	47.7± 3.6

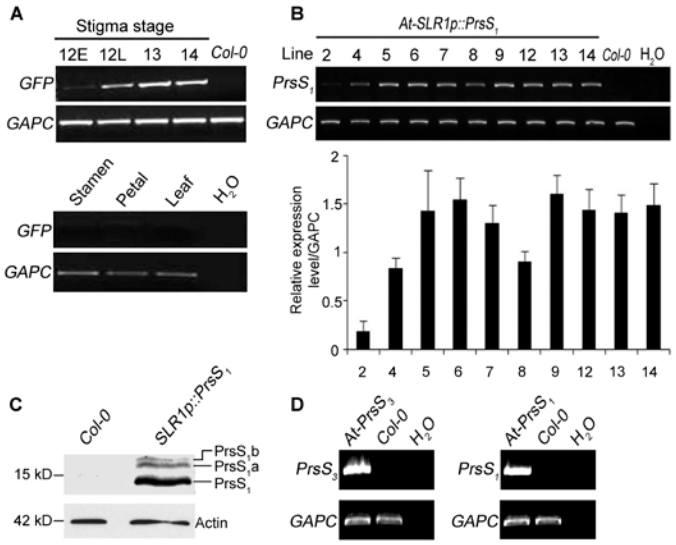

Fig 1.

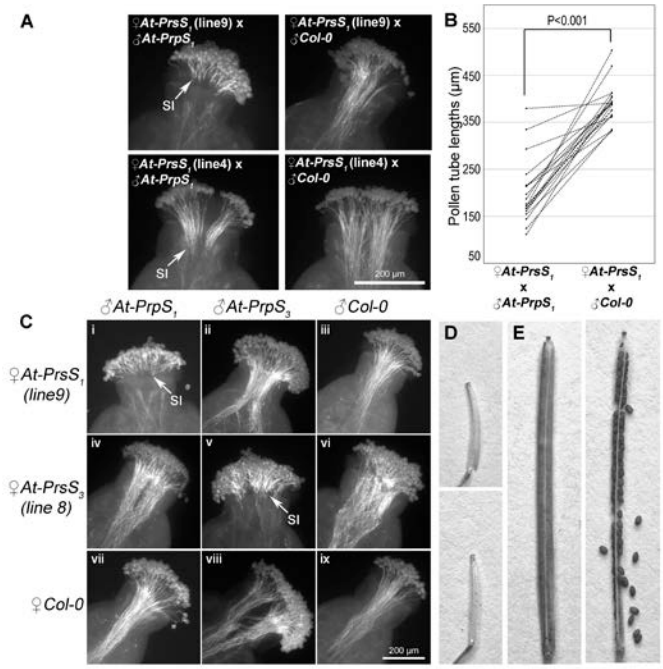
A
♀At-PrsS1 (line9) x ♂At-PrpS1
♀At-PrsS1 (line9) x ♂Col-0
♀At-PrsS1 (line4) x ♂At-PrpS1
♀At-PrsS1 (line4) x ♂Col-0
SI
200 µm
B
P<0.001
Pollen tube lengths (µm)
550
450
350
250
150
50
♀At-PrsS1 x ♂At-PrpS1
♀At-PrsS1 x ♂Col-0
C
♂At-PrpS1
♂At-PrpS3
♂Col-0
♀At-PrsS1 (line9)
♀At-PrsS3 (line 8)
♀Col-0
i
ii
iii
iv
v
vi
vii
viii
ix
SI
200 µm
D
E

Fig 2.

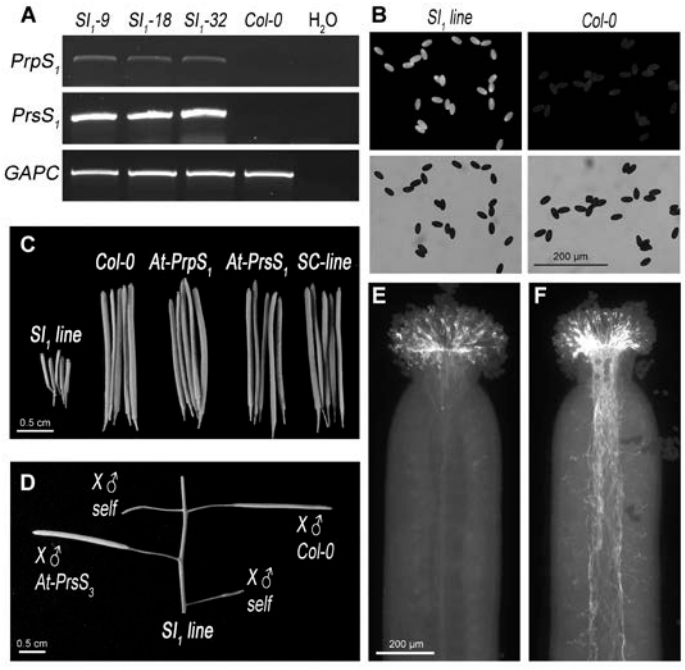
A
SI1-9
SI1-18
SI1-32
Col-0
H2O
PrpS1
PrsS1
GAPC
B
SI1 line
Col-0
200 µm
C
Col-0
At-PrpS1
At-PrsS1
SC-line
SI1 line
0.5 cm
D
X♂ self
X♂ Col-0
X♂ At-PrsS3
X♂ self
SI1 line
0.5 cm
E
200 µm
F

Fig 3.