The Papaver rhoeas S determinants confer self-incompatibility to Arabidopsis thaliana in planta
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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 F₁ 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 *PrsS1* in *Arabidopsis* by transforming a *SLR1p::PrsS1*
construct into Col-0 (At-PrsS1 lines). RT-PCR analysis of pistil mRNA from 10 independent lines revealed differing PrsS1 transcript levels (Fig 1B). Western analysis of pistil extracts confirmed the presence of PrsS1 (Fig 1C). We also transformed Col-0 with a SLR1p::PrsS3 construct to make At-PrsS3 lines. RT-PCR analysis revealed similar transgene expression to the highest-expressing At-PrsS1 line (Fig 1D).

To test the functionality of the At-PrsS1 lines we performed semi-vivo pollination assays (Fig S2) on excised pistils from the At-PrsS1 lines, using pollen from an A. thaliana line expressing PrpS1-GFP (8), referred to as At-PrpS1 hereafter, examining the ability of At-PrsS1 stigmas to inhibit At-PrpS1 ("incompatible") pollen tube growth.

At-PrsS1 line 9 pistils inhibited At-PrpS1 pollen tubes more strongly than line 4, while Col-0 pollen was not inhibited (Fig 2A). Quantitation revealed that in At-PrsS1 pistils, At-PrpS1 pollen tubes were significantly shorter than Col-0 pollen tubes (P<0.001, t-test, n=40; Fig 2B). After 70 min on At-PrsS1 pistils, At-PrpS1 pollen tubes from eight out of ten lines were <300 μm; Col-0 controls were >300 μm (n=40; Fig 2B). Thus, At-PrsS1 pistils support pollen tube growth, but reject At-PrpS1 pollen. At 110 min, At-PrpS1 pollen tubes in At-PrsS1 pistils remained shorter than controls (Fig S3). The level of inhibition of At-PrpS1 pollen tubes in At-PrsS1 pistils correlated with PrsS1 expression levels (Fig S4). This provides strong evidence that PrsS1 functions in A. thaliana pistils to inhibit At-PrpS1 pollen.

A key feature of SI is S-allele specific inhibition of pollen. To test this, we pollinated excised At-PrsS1 or At-PrsS3 pistils with At-PrpS1 pollen or pollen from a line expressing PrpS3-GFP (8),
referred to as \textit{At-PrpS}$_3$ hereafter (Fig 2C). Strong pollen tube inhibition was observed only with cognate combinations of \textit{At-PrsS} with \textit{At-PrpS} (Fig 2C, i, v). Pollinations using non-cognate combinations of \textit{At-PrsS} with \textit{At-PrpS} resulted in normal pollen tube growth (Fig 2C, ii, iv). Controls (Fig 2C, iii, vi, vii, viii, ix) had long pollen tubes. \textit{In vivo} pollinations of \textit{At-PrsS} pistils also revealed differential inhibition of pollen tubes after 18 h (Fig S5). This demonstrates \textit{S}-specific pollen tube inhibition by \textit{A. thaliana} expressing PrsS.

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

Analyzing self-seed set, many siliques were completely empty (7/10 for \textit{At-PrsS}$_1$ pollinated with \textit{At-PrpS}$_1$ and 6/10 for \textit{At-PrsS}$_3$ pollinated with \textit{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, ***, \textit{t-test}, n=10). Pollinations between \textit{Col-0} pistils and \textit{At-PrpS}$_1$ or \textit{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 PrsS₁ and PrpS₁ (SI₁-lines) by transforming homozygous At-PrpS₁-GFP plants with SLR1p::PrsS₁. Lines co-expressing PrsS₃ and PrpS₁ (SC-lines) were also generated. Expression of PrsS₁ and PrpS₁ was examined in three SI₁-lines (Fig 3A). Fluorescence microscopy of pollen from these SI₁-lines confirmed the expression of PrpS₁-GFP (Fig 3B). The SI₁- and SC-lines had a similar vegetative phenotype to Col-0, At-PrpS₁ and At-PrsS₁ plants (Fig S7). However, when left to set self-seed naturally, the SI₁-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-PrpS₁ (16.3 ±1.0), At-PrsS₁ (15.9 ±0.5) and SC plants (15.3 ±0.5 mm; p<0.001 ***, t-test; n=10 per plant). Twelve of the SI₁-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-PrpS₁ plants (57.7 ±2.8), At-PrsS₁ plants (58.3 ±1.6) and SC-lines (57.1 ±1.7; n=10). Total self seed-set from these SI₁-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 PrsS₃ and PrpS₃ (SI₃-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 SI₁-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 SI1-lines (SI1-9, SI1-18 and SI1-32) were pollinated with At-PrpS3 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 SI1-lines was also pollinated to At-PrpS1 stigmas. They produced siliques and seed set not significantly different from At-PrpS1 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.
REFERENCES & NOTES:


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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 PrsS1 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-PrsS1 lines shows expression of PrsS1 (top) and quantitation of PrsS1 expression relative to GAPC (n=3, ± S.D., below).
(C) Western blot (α-PrsS1 antisera) shows expression of PrsS1 in At-PrsS1.
(D) RT-PCR: expression of PrsS3 in At-PrsS3 line 8 is comparable to At-PrsS1 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-PrsS1 pistils with At-PrpS1 or Col-0 pollen.
(B) Quantitation of pollen tube lengths on At-PrsS1 pistils using At-PrpS1 pollen (left) or Col-0 pollen (right); n= 4 stigmas/At-PrsS1 line.
(C) At-PrsS1 and At-PrsS3 pistils pollinated semi-in-vivo with At-PrpS3 or At-PrpS1 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-PrsS1 stigma with At-PrpS1 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 PrsS1 and PrpS1 are self-incompatible and set no seed.
(A) RT-PCR of 3 A. thaliana SI1-lines co-expressing PrsS1 and PrpS1.
(B) Pollen from SI1-lines exhibits GFP fluorescence (top); Col-0 pollen has weak autofluorescence.
(C) Self-seed set: SI1-lines formed short siliques; controls, including a SC-line co-expressing PrsS1 and PrpS1-GFP, set normal siliques
(D) A selfed SI1-plant gave small siliques; pollinations with Col-0 or At-PrpS3 pollen gave normal siliques.
(E) Aniline blue staining of a self-pollinated SI1-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* stigmas with *At-PrpS* pollen resulted in short siliques and reduced seed number, as did pollination of *At-PrsS* with *At-PrpS* pollen. Other control pollinations: non-cognate pollination of *At-PrsS* stigmas with *At-PrpS* pollen, *At-PrsS* stigmas with *At-PrpS* or *Col-0* pollen, *Col-0* stigmas with *Col-0*, *At-PrpS* or *At-PrpS* pollen gave normal siliques and seed number (mean ±S.D., n=10).

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<th><em>At-PrpS</em>&lt;sub&gt;1&lt;/sub&gt;</th>
<th><em>At-PrpS</em>&lt;sub&gt;3&lt;/sub&gt;</th>
<th><em>Col-0</em></th>
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<td>Seeds per silique</td>
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<td><em>At-PrsS</em>&lt;sub&gt;3&lt;/sub&gt;*&lt;sub&gt;(line 8)&lt;/sub&gt;</td>
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<td>Seeds per silique</td>
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