The Papaver Self-Incompatibility Pollen S-Determinant, PrpS, Functions in Arabidopsis thaliana

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Summary

Many angiosperms use specific interactions between pollen and pistil proteins as “self” recognition and/or rejection mechanisms to prevent self-fertilization. Self-incompatibility (SI) is encoded by a multiallelic S locus, comprising pollen and pistil S-determinants [1, 2]. In Papaver rhoes, cognate pistil and pollen S-determinants, PrpS, a pollen-expressed transmembrane protein, and PrsS, a pistil-expressed secreted protein [3, 4], interact to trigger a Ca2+-dependent signaling network [5–10], resulting in inhibition of pollen tube growth, cytoskeletal alterations [11–13], and programmed cell death (PCD) [14, 15] in incompatible pollen. We introduced the PrpS gene into Arabidopsis thaliana, a self-compatible model plant. Exposing transgenic A. thaliana pollen to recombinant Papaver PrsS protein triggered remarkably similar responses to those observed in incompatible Papaver pollen: S-specific inhibition and hallmark features of Papaver SI [11–15]. Our findings demonstrate that Papaver PrpS is functional in a species with no SI system that diverged ~140 million years ago [16]. This suggests that the Papaver SI system utilizes cellular targets that are, perhaps, common to all eudicots and that endogenous signaling components can be recruited to elicit a response that most likely never operated in this species. This will be of interest to biologists interested in the evolution of signaling networks in higher plants.

Results and Discussion

Expression of PrpS-GFP Is Sufficient to Allow PrsS-Induced S-Specific Inhibition of AtPpS Pollen

To determine whether PrpS was functional in A. thaliana, we adapted the in vitro self-incompatibility (SI) bioassay system used for Papaver SI [3]. Transgenic pollen from lines AtPpS1/AtPpS3 was grown in vitro and recombinant Papaver PrsS proteins added. If PrpS functions and utilizes a similar signaling network in Arabidopsis, this interaction should trigger S-specific pollen inhibition in pollen expressing PrpS-GFP. We tested whether this was the case (Figures 1F and 1G). Recombinant PrsS did not affect Col-0 pollen germination but reduced pollen germination from hemizygous AtPpS1 pollen by 42% (n = 300). When only pollen expressing GFP was assessed after addition of PrsS, none of these pollen grains germinated (Figures 1F and 1G). Recombinant PrsS inhibits AtPpS pollen expressing PrsS-GFP. This suggests that expression of PrpS in Arabidopsis pollen is sufficient to allow inhibition of pollen germination by PrsS. Using Papaver pollen (from plants haplotype S3S3) confirmed that PrsS was functional (Figure 1F). Addition of PrsS partially reduced germination (p = 0.022, n = 300), addition of both PrsS1 and PrsS3 achieved complete inhibition (p = 0.009, n = 300).

We next tested lines AtPpS1 and AtPpS3 homozygous for PrpS-GFP expression for S-specific inhibition of pollen tube growth by adding PrsS or PrsS2 (Figure 2). Col-0 pollen tube lengths were not significantly different from untreated transgenic lines after addition of PrsS or PrsS2 (p = 0.87, 0.89, n = 120). When PrsS3 was added to AtPpS1 pollen, pollen tubes were significantly inhibited (>95% shorter compared to untreated controls, ***p < 0.0001, n = 120). Similar results were obtained for PrsS3 addition to AtPpS3 pollen (**p < 0.0001; Figure 2). Inhibition of transgenic pollen was S-allelespecific, as when PrsS3 was added to AtPpS1 pollen, no inhibition was observed compared to untreated controls (p = 0.95, n = 120); likewise, when PrsS3 was added to AtPpS3 pollen, pollen tube lengths were not significantly different from untreated controls (p = 0.66, n = 120, Figure 2). Heat-denatured (biologically inactive) PrsS proteins had no effect. These data are consistent with the idea that PrpS expression in A. thaliana pollen is sufficient for an SI response (inhibition of “self” pollen) to be elicited. Control Papaver pollen from plants with haplotypes S3S3 was inhibited (96% shorter than untreated, n = 120; ***p < 0.0001) after addition of PrsS3 and PrsS3 (Figure 2).
**A. thaliana** Pollen Expressing PrpS-GFP Exhibits S-Specific Actin Alterations after Addition of PrsS

We next investigated whether expression of PrpS in **A. thaliana** pollen was sufficient to induce similar intracellular responses to those elicited in incompatible **Papaver** pollen [7] by adding incompatible recombinant PrsS. A hallmark feature of **Papaver** SI is the S-specific formation of punctate actin foci [11, 12]. Punctate actin foci were formed when PrsS1 was added to **AtPpS1** pollen (Figure 3A); a similar response was observed in **AtPpS3** pollen after addition of PrsS3 (Figure 3B). Untreated pollen from these lines had normal filamentous actin organization (Figures 3C and 3D), and they retained this actin configuration after addition of compatible combinations of PrsS (**AtPpS1** with PrsS8, Figure 3E; **AtPpS3** with PrsS1, Figure 3F).

When heat-denatured PrsS were used in an incompatible combination (Figures 3G and 3H), no actin foci were formed. Untransformed Col-0 pollen exhibited normal actin configuration (Figure 3I), and when PrsS1 was added to this pollen, no foci were formed (Figure 3J). This demonstrates that PrsS affects actin organization of **AtPpS1** and **AtPpS3** pollen.

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**Figure 1.** Expression of PrpS in Transgenic Arabidopsis thaliana

(A) Fifty percent of pollen grains in **A. thaliana** lines **AtPpS1** hemizygous for PrpS1-GFP expression exhibit GFP fluorescence (left); brightfield image, right.

(B) GFP fluorescence is observed in all pollen grains in homozygous **A. thaliana** **AtPpS1** line (left); brightfield image, right.

(C) No GFP fluorescence is observed in **A. thaliana** wild-type pollen grains (left); brightfield image, right.

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**Figure 2.** S-Specific Inhibition of Pollen Tube Growth in **A. thaliana** Pollen Expressing PrpS-GFP by Addition of Cognate PrsS

Pollen tube lengths from homozygous lines **AtPpS1** and **AtPpS3** were measured after addition of PrsS (**AtPpS1** with PrsS8, Figure 3E; **AtPpS3** with PrsS1, Figure 3F). When heat-denatured PrsS were used in an incompatible combination (Figures 3G and 3H), no actin foci were formed. Untransformed Col-0 pollen exhibited normal actin configuration (Figure 3I), and when PrsS1 was added to this pollen, no foci were formed (Figure 3J). This demonstrates that PrsS affects actin organization of **AtPpS1** and **AtPpS3** pollen.
specifically when used in a cognate allelic combination. Quantification (Figures 3K and 3L) showed that filamentous actin is the predominant phenotype, except for the combination of cognate recombinant PrsS with PrpS pollen (AtPpS1 pollen with PrsS1 added, and AtPpS3 pollen with PrsS3). These two samples were significantly different from untreated pollen (**p < 0.0001, n = 250; ***p < 0.0001, n = 350). All other comparisons were not significantly different from untreated controls or Col-0, for example, AtPpS1 pollen with PrsS3 added, compared to untreated pollen (p = 0.85, n = 250). Thus, formation of punctate actin foci is induced in an S-allele-specific manner in Arabidopsis PrpS-expressing pollen by Papaver PrsS. As expression of PrpS in A. thaliana pollen is sufficient to elicit this key hallmark feature of Papaver SI, it suggests that all the signaling components necessary for this “Papa-

S-Specific Death Induced by PrsS in A. thaliana pollen 

Expessing PrpS-GFP

A key feature of SI in Papaver rhoeas is the triggering of programmed cell death (PCD) in incompatible pollen [14, 15]. To provide further evidence for PrpS elicitation of a Papaver-like SI response, we investigated whether death was triggered in AtPpS1 and AtPpS3 pollen after addition of PrsS, by assessing viability of pollen using Evans blue at 8 hr (Figure 4A). PrsS1 and PrsS3 activity was demonstrated by addition to Papaver pollen from plants haplotype S1S3; this gave an 89% loss of viability compared to untreated pollen (**p < 0.0001, n = 300, Figure 4A). Untransformed Col-0 pollen viability was not significantly affected after addition of PrsS1 or PrsS3 (p = 0.71, p = 0.60, n = 500). Addition of PrsS1 to AtPpS1 pollen resulted in a 60% reduction in pollen viability compared to untreated controls (**p < 0.0001, n = 500). Similar results were obtained with PrsS3 added to AtPpS3 pollen (p < 0.0001, n = 500). Loss of viability was S-allele-specific; when PrsS3 was added to AtPpS1 pollen, and when PrsS1 was added to AtPpS3 pollen, there was no significant difference in viability compared to untreated pollen (p = 0.48, 0.83 respectively, n = 500). As expected, heat-denatured PrsS had no effect. Thus, PrsS can trigger S-specific death in A. thaliana pollen expressing PrpS-GFP, specifically in combination with cognate (“self”) PrsS.

S-Specific Death Induced by PrsS Involves a DEVDase/caspase-3-like Activity

Although Evans blue demonstrates cell death, it does not indicate whether PCD is involved. As Papaver SI relies on a DEVDase/caspase-3-like activity [14, 15], we assessed whether a similar activity was involved in the death of PrpS-expressing A. thaliana pollen, by adding Ac-DEVD-CHO, a caspase-3 inhibitor before addition of PrsS (Figure 4B). PrsS1 and PrsS3 added to Papaver pollen carrying PrpS1 and PrpS3 resulted in 91% loss in viability compared to untreated pollen (**p < 0.0001, n = 300); pretreatment with Ac-DEVD-CHO resulted in significantly higher viability at 8 hr (p < 0.0001, n = 300). Ac-DEVD-CHO had no effect on Arabidopsis pollen viability (p = 0.66 for Col-0, p = 0.60 for AtPpS1, 0.23 for AtPpS3). Pretreatment of pollen with Ac-DEVD-CHO before PrsS addition resulted in significantly higher viability compared to samples with PrsS1 or PrsS3 added alone. AtPpS1 pollen viability was not significantly different to that in the presence of Ac-DEVD-CHO alone (p = 0.065, NS, n = 300); for AtPpS3 homozygotes, viability was only 17% less than pollen from the same line in the presence of Ac-DEVD-CHO alone (p = 0.13, NS, n = 300).
Prevention of PrsS-induced death of AtPpS1 and AtPpS3 pollen by Ac-DEVD-CHO provides strong evidence that PrpS triggers a functional "Papaver-like" SI response involving a DEVDase/caspase-3-like activity in A. thaliana pollen. It also suggests that similar signaling networks to those used in the Papaver SI response [14, 15, 17] are used in AtPpS pollen that result in pollen PCD.

Together, our findings demonstrate that although the SI determinants in Papaver are completely distinct from those identified at a molecular level in other SI systems, PrpS functions in A. thaliana pollen in a S-specific manner. This provides good evidence that A. thaliana has all the components required for a Brassica-type SI to be elicited, though the detailed mechanisms are not yet fully elucidated. Although these are important demonstrations, A. thaliana and A. lyrata diverged only ~5 million years ago (mya) [21], Arabidopsis and Capsella separated ~6.2–9.8 mya [25], and self-compatibility originated very recently (<0.5 mya) [26]. Thus, despite the importance of these studies, major insights into the evolution of SI signaling across angiosperm families is lacking as a result of their close relationship and their possession of a mechanistically common SI system. P. rhoeas has a gametophytic SI system that is genetically controlled in a completely different manner from the sporophytic SI system in the Brassicaceae. These two SI systems are thought to have evolved completely independently [27], and there is no evidence of a shared ancestral SI system, because A. thaliana does not possess orthologs of the Papaver S-determinants. Here we show that, despite the huge evolutionary distance and lack of a common SI system, transgenic A. thaliana pollen expressing PrpS-GFP...
is not only rejected but also displays remarkably similar cellular responses to that triggered in incompatible *Papaver* pollen.

Our data provide good evidence that *A. thaliana* recruits existing proteins to form new signaling networks to trigger a function (SI) that does not normally operate in this species. As a *Papaver*-like SI response, involving formation of punctate actin foci and PCD involving a caspase-3-like DEVDase activity has not been observed in the *Brassica*-type SI response, it suggests that the PrpS-PrsS interaction is sufficient to specify a particular downstream signaling network to obtain this outcome. Studies on the evolution of self-nonself-recognition systems has largely focused on the receptors and ligands involved in recognition [28, 29] rather than the signaling networks triggered by their interaction. Our findings suggest either conservation of a signaling system or recruitment of core signaling components to mediate downstream SI responses and will open up debate about how these systems evolved. It appears that the *Papaver* SI system works in *A. thaliana* due to “multitasking” of endogenous components that can “plug and play” to act in signaling networks that they do not normally operate in, to provide a specific, predictable physiological outcome. This has previously been shown in other systems (see [30–32]), and a compelling argument has been made for the utilization of convergent evolution in innate immune pathways [33]. Our findings confirm postulated parallels between SI and plant-pathogen resistance [29, 34] and the idea that SI may utilize these signaling networks. Our data suggest that the signaling networks and cellular targets for *Papaver* SI are “universal,” unspecialized, and ancient and may be present in a wide range of angiosperm species. We suggest that this is a likely explanation of why PrpS functions in *A. thaliana* pollen.

**Conclusions**

Expression of the *Papaver* male S-determinant, PrpS, in *A. thaliana* pollen is sufficient to allow it to differentiate between different allelic products of the *Papaver* female S-locus determinant, PrsS, and trigger an S-allele specific rejection response when it encounters cognate PrsS protein. Functionality in a highly diverged compatible species has implications for our perspective of evolution of signaling networks in higher plants. Moreover, wide transgenera functionality of the *Papaver* SI system opens up the possibility that, assuming that PrsS can also be functionally expressed, transferral of these S-determinants may, in the longer-term, provide a tractable SI system to transfer to crop plants. This has implications for solving food security issues, by allowing breeding of superior F1 hybrid plants more easily and cheaply.

**Supplemental Information**

Supplemental Information includes two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.12.006.

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