Current Biology 22, 154–159, January 24, 2012 ©2012 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.12.006

Report

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

Barend H.J. de Graaf,[1,2,5] Sabina Vatovec,[1,5] Javier Andrés Juárez-Díaz,[1,5] Lijun Chai,[1] Kreepa Kooblall,[1,3] Katie A. Wilkins,[1] Huawen Zou,[1,4] Thomas Forbes,[1] F. Christopher H. Franklin,[1,6] and Vernonica E. Franklin-Tong[1,6,*]

[1]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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 rhoeas*, 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 Ca^{2+}-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 uses 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 in *Arabidopsis thaliana* Pollen

Transgenic lines from self-compatible *A. thaliana* ecotype Columbia (Col-0) were generated by introducing *$PrpS_1$-GFP* (line *AtPpS1*) or *$PrpS_3$-GFP* (line *AtPpS3*) under the control of the pollen-specific promoter *ntp303p* (see Supplemental Experimental Procedures available online). Transgenic lines in the T_2 generation that segregated 3:1 were identified and pooled pollen assessed for GFP-expression. Two-thirds of the pollen was expected to be GFP-positive; 63.5% GFP expression was observed (n = 300). When pollen from individual plants was analyzed, pollen segregated either 50% or 100% for GFP-expression (n = 2,000, Figures 1A and 1B), consistent with them being hemizygous or homozygous for the insert; untransformed Col-0 pollen had low autofluorescence (Figure 1C). PrpS-GFP localized predominantly at the plasma membrane in pollen tubes (Figure 1D) as previously shown in *Papaver* pollen [4]. Expression of the *$PrpS_1$/$PrpS_3$* transgenes in these lines was confirmed using RT-PCR; transcripts were not detected in untransformed Col-0 plants (Figure 1E).

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 $PrsS_1$ 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 $PrsS_1$, none of these pollen grains germinated (Figures 1F and 1G, ***$p < 0.0001$, n = 300). This correlation of GFP expression and pollen inhibition by $PrsS_1$ demonstrates that $PrsS_1$ inhibits *AtPpS1* pollen expressing $PrpS_1$-GFP. This suggests that expression of $PrpS_1$ in *Arabidopsis* pollen is sufficient to allow inhibition of pollen germination by $PrsS_1$. Using *Papaver* pollen (from plants haplotype *S_1S_8*) confirmed that $PrsS_1$ was functional (Figure 1F). Addition of $PrsS_1$ partially reduced germination ($p = 0.022$, n = 300), addition of both $PrsS_1$ and $PrsS_8$ 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 $PrsS_1$ or $PrsS_3$ (Figure 2). Col-0 pollen tube lengths were not significantly different from untreated transgenic lines after addition of $PrsS_1$ or $PrsS_3$ ($p = 0.87, 0.89$, n = 120). When $PrsS_1$ 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 $PrsS_3$ addition to *AtPpS3* pollen (***$p < 0.0001$; Figure 2). Inhibition of transgenic pollen was *S*-allele-specific, as when $PrsS_3$ was added to *AtPpS1* pollen, no inhibition was observed compared to untreated controls ($p = 0.95$, n = 120); likewise, when $PrsS_1$ 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 *S_1S_3* was inhibited (96% shorter than untreated, n = 120; ***$p < 0.0001$) after addition of $PrsS_1$ and $PrsS_3$ (Figure 2).

[2]Present address: School of Biosciences, Cardiff University, Cardiff CF10 3AT, UK
[3]Present address: Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK
[4]Present address: Yangtze University, Jingzhou, Hubei 434025, China
[5]These authors contributed equally to this work
[6]These authors contributed equally to this work
*Correspondence: v.e.franklin-tong@bham.ac.uk

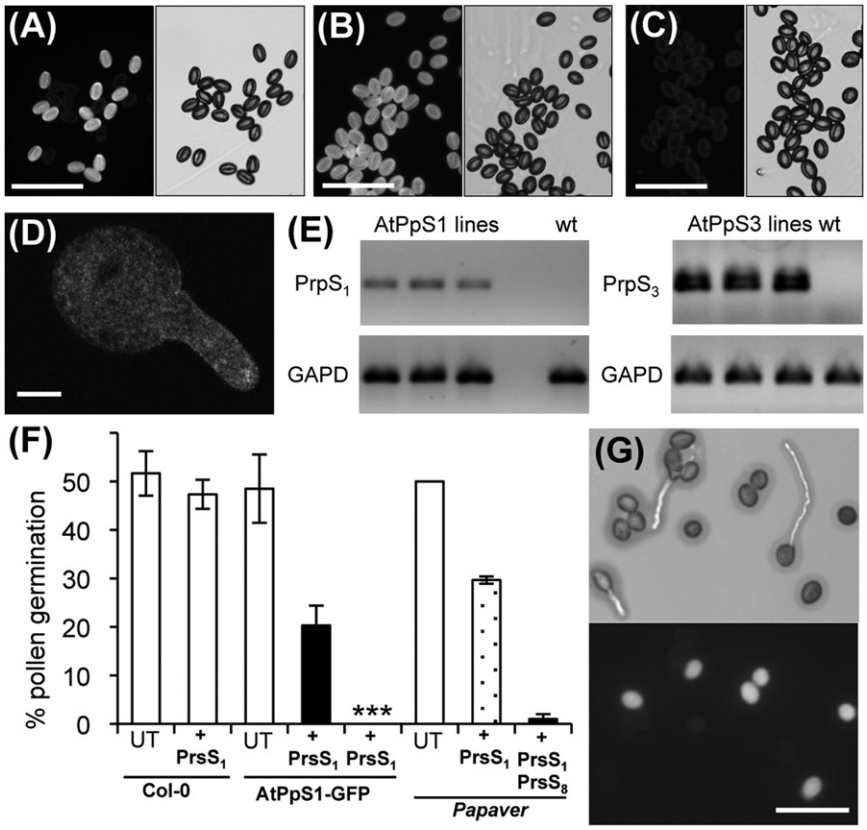

Figure 1. Expression of PrpS in Transgenic *Arabidopsis thaliana*

(A) Fifty percent of pollen grains in *A. thaliana* lines *AtPpS1* hemizygous for $PrpS_1$-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.

(D) Confocal image of a $PrpS_1$-GFP-expressing pollen tube.

(E) RT-PCR to show expression of PrpS in *A. thaliana AtPpS1* and *AtPpS3* lines; WT, wild-type Col-0; GAPD was a loading control.

(F) Quantification of inhibition of pollen germination of a hemizygous line of *AtPpS1* by $PrsS_1$. Control pollen had high germination (white bars): untreated (UT), Col-0 pollen was unaffected by addition of $PrsS_1$ (+$PrsS_1$). Addition of $PrsS_1$ to hemizygous GFP-expressing *AtPpS1* pollen (+$PrsS_1$) had reduced pollen germination (black bar). When only GFP-expressing pollen were measured for this latter treatment (+$PrsS_1$), no germination was observed (***). *Papaver* pollen (from a plant haplotype S_1S_8): untreated (UT) had high germination, addition of $PrsS_1$ inhibited half of the pollen, and addition of $PrsS_1$ and $PrsS_8$ gave inhibition of all pollen.

(G) Pollen grains from a hemizygous *AtPpS1* line. Those not expressing $PrpS_1$-GFP germinate and grow in the presence of $PrsS_1$, whereas those exhibiting GFP fluorescence do not.

Scale bars in (A), (B), (C), and (G) represent 100 μm; scale bar in (D) represents 10 μm. Error bars indicate ±SEM.

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 $PrsS_1$ was added to *AtPpS1* pollen (Figure 3A); a similar response was observed in *AtPpS3* pollen after addition of $PrsS_3$ (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 $PrsS_8$, Figure 3E; *AtPpS3* with $PrsS_1$, 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 $PrsS_1$ was added to this pollen, no foci were formed (Figure 3J). This demonstrates that PrsS affects actin organization of *AtPpS1* and *AtPpS3* pollen

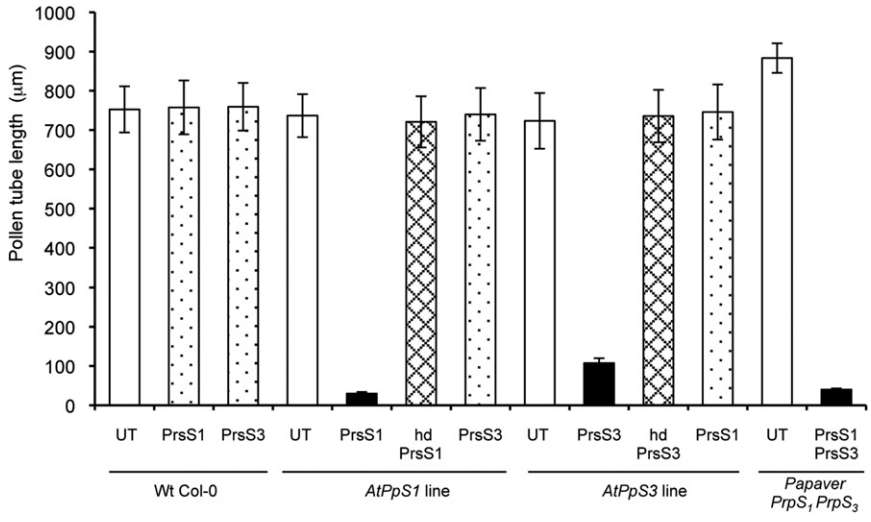

Figure 2. *S*-Specific Inhibition of Pollen Tube Growth in *A. thaliana* Pollen Expressing PrpS-GFP by Addition of Cognate PrsS

Pollen tube lengths from homozygous lines *AtPpS1* and *AtPpS3* were measured after addition of $PrsS_1$ and $PrsS_3$. Untreated pollen tubes (UT, white bars) grew long; $PrsS_1$ specifically inhibited pollen from line *AtPpS1* (black bar) and not pollen from *AtPpS3* or Col-0 (speckled bars); $PrsS_3$ specifically inhibited pollen from line *AtPpS3* (black bar) and not pollen from *AtPpS1* or Col-0 (speckled bars). Heat-denatured PrsS (hd; cross-hatched bars) had no effect on pollen tube length. Untreated *Papaver* pollen from a plant haplotype S_1S_8 (UT, white bar) had long pollen tubes, and addition of $PrsS_1$ and $PrsS_8$ gave strong inhibition (black bar). Error bars indicate ±SEM.

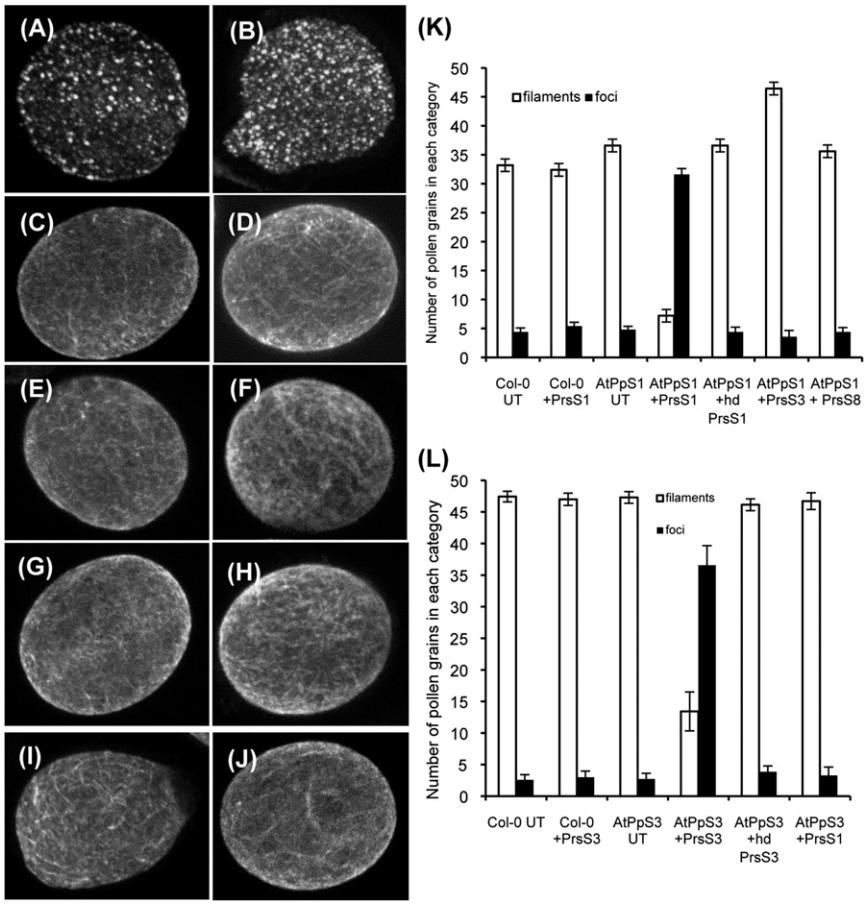

Figure 3. Actin Foci Are Stimulated in an *S*-Specific Manner in *A. thaliana AtPpS* Pollen by Cognate PrsS

(A–J) F-actin was visualized using rhodamine-phalloidin and confocal imaging.

(A and B) Typical punctate actin foci observed 3 hr after addition of $PrsS_1$ to an *AtPpS1* pollen grain (A) and $PrsS_3$ to an *AtPpS3* pollen grain (B).

(C–F) Controls with normal actin arrays: untreated *AtPpS1* (C) and untreated *AtPpS3* pollen grains (D); "compatible" combinations (E and F), $PrsS_8$ added to an *AtPpS1* pollen grain (E), $PrsS_1$ added to an *AtPpS3* pollen grain (F), and heat-denatured $PrsS_1$ and $PrsS_3$ did not induce actin foci in *AtPpS1* and *AtPpS3* respectively (G and H).

(I and J) Normal actin arrays were observed in wild-type Col-0 pollen grain untreated (I) or after addition of $PrsS_1$ (J).

(K and L) Quantitation of F-actin foci and normal filamentous actin arrays in pollen from the *A. thaliana AtPpS1* lines (K), pollen from the *A. thaliana AtPpS3* lines (L), and Col-0 acted as a control. White bars show normal actin filament arrays (as in C–F); black bars show punctate actin foci (as in A and B). Error bars indicate ±SEM.

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 $PrsS_1$ added, and *AtPpS3* pollen with $PrsS_3$). 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 $PrsS_8$ 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 "*Papaver*-like" SI response are present.

S-Specific Death Is Induced by PrsS in *A. thaliana* pollen Expressing 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). $PrsS_1$ and $PrsS_3$ activity was demonstrated by addition to *Papaver* pollen from plants haplotype S_1S_3; 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 $PrsS_1$ or $PrsS_3$ ($p = 0.71$, $p = 0.60$, $n = 500$). Addition of $PrsS_1$ 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 $PrsS_3$ added to *AtPpS3* pollen ($p < 0.0001$, $n = 500$). Loss of viability was *S*-allele-specific; when $PrsS_3$ was added to *AtPpS1* pollen, and when $PrsS_1$ 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). $PrsS_1$ and $PrsS_3$ added to *Papaver* pollen carrying $PrpS_1$ and $PrpS_3$ 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 $PrsS_1$ or $PrsS_3$ 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, $n = 300$).

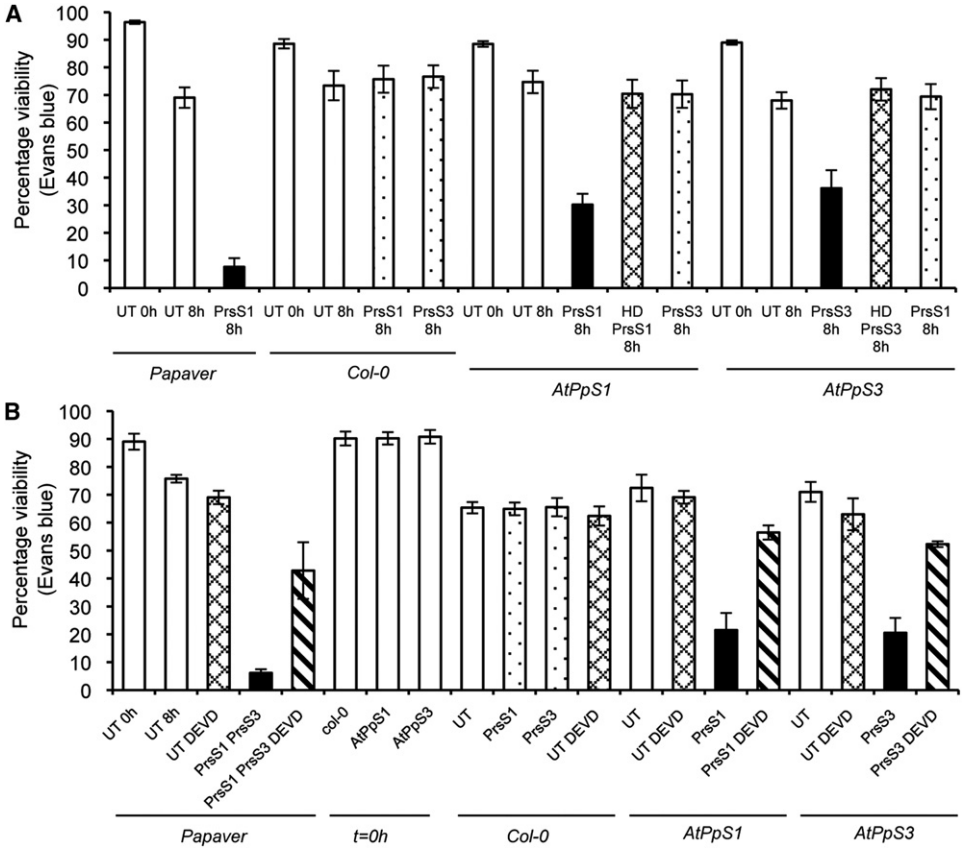

Figure 4. Death Involving a DEVDase/caspase-3-like Activity Is Stimulated in an *S*-Specific Manner in *A. thaliana* Expressing $PrpS_1$-GFP or $PrpS_3$-GFP

(A) Quantitation of Evans blue staining 8 hr after addition of PrsS (percent viability). All untreated (UT, white bars) pollen at time 0 had high viability; this was slightly reduced after 8 hr. Addition of $PrsS_1$ and $PrsS_3$ to *Papaver* pollen carrying $PrpS_1$ and $PrpS_3$ resulted in low viability (black bar); addition of PrsS (speckled bars) to *A. thaliana* Col-0 pollen did not affect viability; addition of $PrsS_1$ to *AtPpS1* pollen and $PrsS_3$ to *AtPpS3* pollen reduced viability (black bars). Heat-denatured PrsS (HD-PrsS, cross-hatched bars) did not affect viability.

(B) Pretreatment with Ac-DEVD-CHO prevents *S*-specific death of *A. thaliana* pollen. Quantitation of percent viability (Evans blue) after pretreatment with Ac-DEVD-CHO and addition of PrsS. Untreated (UT, white bars) pollen had high viability. Addition of the caspase-3 inhibitor, Ac-DEVD-CHO to UT pollen (UT DEVD, cross-hatched) had no effect. Addition of $PrsS_1$ and $PrsS_3$ to *Papaver* pollen carrying $PrpS_1$ and $PrpS_3$ resulted in low viability (black), and pretreatment with Ac-DEVD-CHO prior to addition of $PrsS_1$ or $PrsS_3$ (diagonal bars) resulted in higher viability. Addition of PrsS to *A. thaliana* Col-0 pollen did not affect viability (stippled bars); addition of $PrsS_1$ to *AtPpS1* pollen and $PrsS_3$ to *AtPpS3* pollen reduced viability (black bars). Pretreatment with Ac-DEVD-CHO prior to addition of $PrsS_1$ and $PrsS_3$ (diagonal bars) resulted in higher viability.

Error bars indicate ±SEM.

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 as an *S*-determinant when transferred into a self-compatible species from a distantly related genus. *Papaver* belongs to the most basal order in the eudicots, the Ranunculales, whereas *Arabidopsis* belongs to the Brassicales, with ~140 million years evolutionary distance between them [16]; see Figure S1. So far the only functional transfer of *S*-determinants has been between closely related species. Interspecific and intergeneric transfer of orthologs of *Brassica S*-determinants [18–20] from self-incompatible *A. lyrata* and *Capsella grandiflora* [21, 22] into self-compatible *A. thaliana* is sufficient to confer SI [23, 24]. This provided 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-/non-self-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.

Acknowledgments

Work in the laboratories of F.C.H.F. and V.E.F.-T. is funded by the Biotechnology and Biological Sciences Research Council (BBSRC). B.H.J. de G. was supported by BBSRC, J.A.J.-D. by CONACYT-México, H.Z. by a China Scholarship Council scholarship, S.V. and K.A.W. by BBSRC PhD studentships, and L.C. by a scholarship from Huazhong Agricultural University. K.K. and T.F. were undergraduate students. Thanks to Steve Price for technical support with transgenic plants. Thanks to Chuck Bell and Vivien Irish for providing an estimate of divergence between *Papaver* and *Arabidopsis*.

Received: October 16, 2011
Revised: November 24, 2011
Accepted: November 28, 2011
Published online: December 29, 2011

References

1. Takayama, S., and Isogai, A. (2005). Self-incompatibility in plants. Annu. Rev. Plant Biol. *56*, 467–489.
2. Franklin-Tong, V.E., ed. (2008). Self-Incompatibility in Flowering Plants: Evolution, Diversity, and Mechanisms (Berlin, Heidelberg: Springer).
3. Foote, H.C.C., Ride, J.P., Franklin-Tong, V.E., Walker, E.A., Lawrence, M.J., and Franklin, F.C.H. (1994). Cloning and expression of a distinctive class of self-incompatibility (*S*) gene from *Papaver rhoeas* L. Proc. Natl. Acad. Sci. USA *91*, 2265–2269.
4. Wheeler, M.J., de Graaf, B.H.J., Hadjiosif, N., Perry, R.M., Poulter, N.S., Osman, K., Vatovec, S., Harper, A., Franklin, F.C.H., and Franklin-Tong, V.E. (2009). Identification of the pollen self-incompatibility determinant in *Papaver rhoeas*. Nature *459*, 992–995.
5. Franklin-Tong, V.E., Ride, J.P., Read, N.D., Trewavas, A.J., and Franklin, F.C.H. (1993). The Self-Incompatibility response in *Papaver rhoeas* is mediated by cytosolic-free calcium. Plant J. *4*, 163–177.
6. Wu, J., Wang, S., Gu, Y., Zhang, S., Publicover, S.J., and Franklin-Tong, V.E. (2011). Self-incompatibility in *Papaver rhoeas* activates nonspecific cation conductance permeable to Ca^{2+} and K^{+}. Plant Physiol. *155*, 963–973.
7. Bosch, M., and Franklin-Tong, V.E. (2008). Self-incompatibility in *Papaver*: signalling to trigger PCD in incompatible pollen. J. Exp. Bot. *59*, 481–490.
8. Rudd, J.J., Franklin, F.C.H., Lord, J.M., and Franklin-Tong, V.E. (1996). Increased phosphorylation of a 26-kD pollen protein is induced by the self-incompatibility response in *Papaver rhoeas*. Plant Cell *8*, 713–724.
9. de Graaf, B.H.J., Rudd, J.J., Wheeler, M.J., Perry, R.M., Bell, E.M., Osman, K., Franklin, F.C.H., and Franklin-Tong, V.E. (2006). Self-incompatibility in *Papaver* targets soluble inorganic pyrophosphatases in pollen. Nature *444*, 490–493.
10. Rudd, J.J., Osman, K., Franklin, F.C.H., and Franklin-Tong, V.E. (2003). Activation of a putative MAP kinase in pollen is stimulated by the self-incompatibility (SI) response. FEBS Lett. *547*, 223–227.
11. Geitmann, A., Snowman, B.N., Emons, A.M.C., and Franklin-Tong, V.E. (2000). Alterations in the actin cytoskeleton of pollen tubes are induced by the self-incompatibility reaction in *Papaver rhoeas*. Plant Cell *12*, 1239–1251.
12. Poulter, N.S., Staiger, C.J., Rappoport, J.Z., and Franklin-Tong, V.E. (2010). Actin-binding proteins implicated in the formation of the punctate actin foci stimulated by the self-incompatibility response in *Papaver*. Plant Physiol. *152*, 1274–1283.
13. Snowman, B.N., Kovar, D.R., Shevchenko, G., Franklin-Tong, V.E., and Staiger, C.J. (2002). Signal-mediated depolymerization of actin in pollen during the self-incompatibility response. Plant Cell *14*, 2613–2626.
14. Thomas, S.G., and Franklin-Tong, V.E. (2004). Self-incompatibility triggers programmed cell death in *Papaver* pollen. Nature *429*, 305–309.
15. Bosch, M., and Franklin-Tong, V.E. (2007). Temporal and spatial activation of caspase-like enzymes induced by self-incompatibility in *Papaver* pollen. Proc. Natl. Acad. Sci. USA *104*, 18327–18332.
16. Bell, C.D., Soltis, D.E., and Soltis, P.S. (2010). The age and diversification of the angiosperms re-revisited. Am. J. Bot. *97*, 1296–1303.
17. Wilkins, K.A., Bancroft, J., Bosch, M., Ings, J., Smirnoff, N., and Franklin-Tong, V.E. (2011). Reactive oxygen species and nitric oxide mediate actin reorganization and programmed cell death in the self-incompatibility response of *papaver*. Plant Physiol. *156*, 404–416.
18. Takasaki, T., Hatakeyama, K., Suzuki, G., Watanabe, M., Isogai, A., and Hinata, K. (2000). The *S* receptor kinase determines self-incompatibility in *Brassica* stigma. Nature *403*, 913–916.
19. Schopfer, C.R., Nasrallah, M.E., and Nasrallah, J.B. (1999). The male determinant of self-incompatibility in *Brassica*. Science *286*, 1697–1700.
20. Takayama, S., Shiba, H., Iwano, M., Shimosato, H., Che, F.-S., Kai, N., Watanabe, M., Suzuki, G., Hinata, K., and Isogai, A. (2000). The pollen determinant of self-incompatibility in *Brassica campestris*. Proc. Natl. Acad. Sci. USA *97*, 1920–1925.
21. Koch, M.A., Haubold, B., and Mitchell-Olds, T. (2000). Comparative evolutionary analysis of chalcone synthase and alcohol dehydrogenase loci in *Arabidopsis, Arabis*, and related genera (Brassicaceae). Mol. Biol. Evol. *17*, 1483–1498.

22. Paetsch, M., Mayland-Quellhorst, S., and Neuffer, B. (2006). Evolution of the self-incompatibility system in the Brassicaceae: identification of *S*-locus receptor kinase (*SRK*) in self-incompatible *Capsella grandiflora*. Heredity (Edinb) *97*, 283–290.
23. Nasrallah, M.E., Liu, P., and Nasrallah, J.B. (2002). Generation of self-incompatible *Arabidopsis thaliana* by transfer of two *S* locus genes from *A. lyrata*. Science *297*, 247–249.
24. Boggs, N.A., Dwyer, K.G., Shah, P., McCulloch, A.A., Bechsgaard, J., Schierup, M.H., Nasrallah, M.E., and Nasrallah, J.B. (2009). Expression of distinct self-incompatibility specificities in *Arabidopsis thaliana*. Genetics *182*, 1313–1321.
25. Acarkan, A., Rossberg, M., Koch, M., and Schmidt, R. (2000). Comparative genome analysis reveals extensive conservation of genome organisation for *Arabidopsis thaliana* and *Capsella rubella*. Plant J. *23*, 55–62.
26. Bechsgaard, J.S., Castric, V., Charlesworth, D., Vekemans, X., and Schierup, M.H. (2006). The transition to self-compatibility in *Arabidopsis thaliana* and evolution within *S*-haplotypes over 10 Myr. Mol. Biol. Evol. *23*, 1741–1750.
27. Allen, A.M., and Hiscock, S.J. (2008). Evolution and phylogeny of self-incompatibility systems in Angiosperms. In Self-Incompatibility in Flowering Plants, V.E. Franklin-Tong and H. Berlin, eds. (Heidelberg: Springer Berlin), pp. 73–101.
28. Boehm, T. (2006). Quality control in self/nonself discrimination. Cell *125*, 845–858.
29. Sanabria, N., Goring, D., Nürnberger, T., and Dubery, I. (2008). Self/nonself perception and recognition mechanisms in plants: a comparison of self-incompatibility and innate immunity. New Phytol. *178*, 503–514.
30. Eckardt, N.A. (2002). Good things come in threes: a trio of triple kinases essential for cell division in *Arabidopsis*. Plant Cell *14*, 965–967.
31. Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature *415*, 977–983.
32. Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. *79*, 143–180.
33. Ausubel, F.M. (2005). Are innate immune signaling pathways in plants and animals conserved? Nat. Immunol. *6*, 973–979.
34. Hodgkin, T., Lyon, G.D., and Dickinson, H.G. (1988). Recognition in flowering plants: A comparison of the *Brassica* self-incompatibility system and plant pathogen interactions. New Phytol. *110*, 557–569.