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Review

Contrasting self-recognition rejection systems for self-incompatibility in *Brassica* and *Papaver*

Daphne R. Goring[1], Maurice Bosch[2], and Vernonica E. Franklin-Tong[3,*]
[1]Department of Cell & Systems Biology, University of Toronto, Toronto M5S 3B2, Canada
[2]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth SY23 3EB, Wales, UK
[3]School of Biosciences, University of Birmingham, Edgbaston B15 2TT, UK
*Correspondence: v.e.franklin-tong@bham.ac.uk
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SUMMARY

Self-incompatibility (SI) plays a pivotal role in whether self-pollen is accepted or rejected. Most SI systems employ two tightly linked loci encoding highly polymorphic pollen (male) and pistil (female) *S*-determinants that control whether self-pollination is successful or not. In recent years our knowledge of the signalling networks and cellular mechanisms involved has improved considerably, providing an important contribution to our understanding of the diverse mechanisms used by plant cells to recognise each other and elicit responses. Here, we compare and contrast two important SI systems employed in the Brassicaceae and Papaveraceae. Both use 'self-recognition' systems, but their genetic control and *S*-determinants are quite different. We describe the current knowledge about the receptors and ligands, and the downstream signals and responses utilized to prevent self-seed set. What emerges is a common theme involving the initiation of destructive pathways that block the key processes that are required for compatible pollen–pistil interactions.

Introduction

Many plants have hermaphrodite flowers and little control over the pollen that lands on a stigma. Plants have, however, evolved intricate, specialised mechanisms to limit the pollen that fertilises them. Sexual reproduction in higher plants involves pollination, which is followed by complex cell–cell recognition and signalling events between the pollen and a receptive pistil. These play a decisive role in determining reproductive success (see Johnson *et al.*[1] for a review). It has long been recognised that the maintenance of genetic diversity by the prevention of self-fertilisation is hugely advantageous. Charles Darwin made detailed comparative studies of the outcomes of self- and cross-fertilisation and found that selfing had a deleterious effect on the fitness of progeny[2]. Probably the most important mechanism to ensure outcrossing and the prevention of inbreeding depression is self-incompatibility (SI), which is used by ~50% of higher plant species. SI is an ancient process and is thought to be one of the major reasons for the success of angiosperms. Species with functional SI systems diversify at a higher rate than those that are self-compatible, providing evidence for a strong species selection towards SI and helping to explain how this phenomenon has persisted in lineages for at least ~90 million years[3].

SI utilises sophisticated, highly regulated mechanisms during specific pollen–pistil interactions after pollination to ensure recognition and rejection of incompatible (i.e. self) pollen at a specific point in its journey from the stigma, through the pistil to the ovule (Figure 1A; see Broz and Bedinger[4] for a review). The genetics of SI were worked out by pollination studies involving controlled crosses in the early-mid 20th century. This resulted in SI species being classified as having either gametophytic SI (GSI) or sporophytic SI (SSI), based on the genetic control of their pollen SI phenotype. SI is generally controlled by a single *S* locus region, composed of at least two tightly linked loci encoding the pollen (male) and pistil (female) *S*-determinants that exhibit tight tissue-specific and developmental-stage-specific expression. A feature of all SI systems is the extremely polymorphic nature of the *S*-determinants (often <50% amino acid identity between alleles); the number of *S*-haplotypes identified by pollinations in natural populations can be as high as 41[5]. The level of polymorphism has been compared to that of the major histocompatibility complex in animals because of the huge numbers of alleles involved; the nature of the allelic specificity and how this has evolved has long been of interest.

Over the last few decades, our knowledge of the molecular basis of how SI is controlled has expanded considerably. Three different SI systems have been well characterised at a molecular/cellular level to date, with *S*-determinants identified in many species. These comprise an SSI system in the Brassicaceae (Figure 1B) and two very different GSI systems: one in the Papaveraceae (Figure 1C) and one, the S-RNase system, in several families including members of the Solanaceae, Rosaceae, Plantaginaceae and Rutaceae (Figure 1D). The fact that there are three very different SI systems provides strong evidence that SI has evolved independently several times. It is also clear that there are other SI systems in species in which the *S*-determinants are known to be different, but their identity has not yet been established. SI in both the Brassicaceae and the Papaveraceae utilises receptor–ligand-type interactions, triggering downstream signalling networks to inhibit fertilisation by incompatible pollen. In contrast, the S-RNase system uses a completely different approach: toxic ribonucleases enter the pollen and are inactivated in compatible interactions[6,7]. A relatively recent discovery is that there are two different ways of achieving

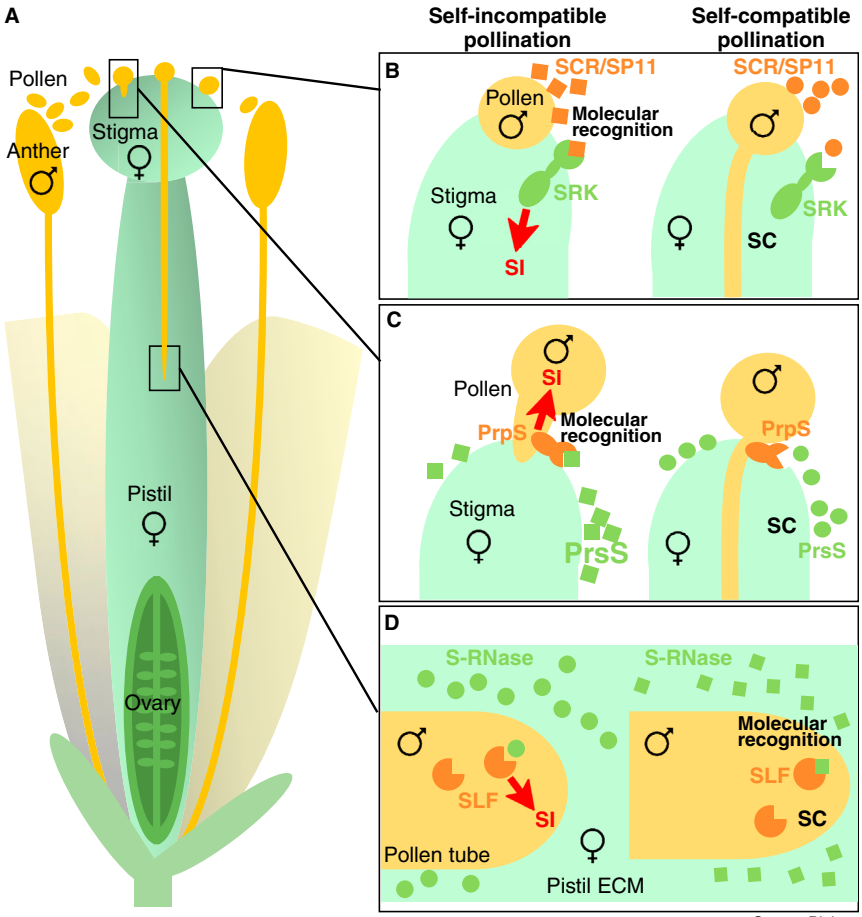

Figure 1. The *S*-determinants of the three best-characterised SI systems and their sites of interaction using self-/non-self-recognition.

(A) Cartoon of a generalised, basic floral morphology. The male anthers shed pollen grains, which are transferred to the stigma. Each pollen adheres, hydrates and germinates, forming a pollen tube that grows through the pistil. The goal is to fertilise an ovule in the ovary. SI prevents fertilisation at different places, depending on the species. The boxes indicate where the SI interactions and pollen inhibition occur for the three best-characterised SI systems. In the Brassicaceae (B) and Papaveraceae (C), inhibition of incompatible pollen takes place on the stigma surface, whereas the *S*-RNase system found in members of the Solanaceae, Rosaceae, Plantaginaceae and Rutaceae (D) causes incompatible pollen tubes to be inhibited as they grow through the pistil. (B–D) The self-incompatible (SI) pollination (left) and self-compatible (SC) pollination (right) scenarios for each of these SI systems. The pistil and female *S*-determinant are indicated in green; the pollen and male *S*-determinant are indicated in orange. (B) In the Brassicaceae, the female *S*-determinant is a receptor kinase, SRK, and the male *S*-determinant is a small, secreted ligand, SCR/SP11. Interaction of cognate SRK and SCR/SP11 occurs at the plasma membrane of stigmatic papilla cells and triggers an SI response in the stigma (left); responses triggered within the stigma result in the rejection of incompatible (self) pollen. In a self-compatible (SC) situation (right), non-cognate *S*-determinants do not interact, and pollen is not inhibited, so pollen germinates and grows a pollen tube. (C) In the Papaveraceae, the female *S*-determinant, PrsS, is a small, secreted ligand and the male *S*-determinant, PrpS, is a transmembrane protein. Interaction of cognate PrsS and PrpS at the pollen plasma membrane (left) triggers intracellular signalling within incompatible (self) pollen, resulting in rejection of self-pollen (SI). In a self-compatible situation (right), non-cognate *S*-determinants do not interact, so pollen is not inhibited. (D) In the *S*-RNase system, the female *S*-determinant (green) is an *S*-RNase that is secreted into the pistil extracellular matrix (ECM), and the male *S*-determinants are F-box proteins, SLFs. In contrast to the systems shown in (B) and (C), interaction of the *S*-determinants occurs within the pollen tube as it grows through the pistil ECM and, generally, a non-self-recognition system operates. The *S*-RNases are taken up into the pollen tube in a non-specific manner. No interaction occurs between 'self' *S*-determinants (left), so RNase toxicity causes failure of incompatible (self) pollen to grow further. In a non-self situation, SLFs interact with non-self *S*-RNases (right), which are detoxified and compatible pollen tubes therefore continue to grow. Note that a self-recognition SI system operates in the Brassicaceae (B) and Papaveraceae (C) and a non-self-recognition system operates in the *S*-RNase system (D).

SI. Intriguingly, while the Brassicaceae and Papaver SI systems utilise a self-recognition system, the S-RNase system employs a non-self-recognition system, with self-recognition occurring in the absence of molecular interactions (Figure 1B–D) and groups of pollen *S*-determinants working together in a 'collaborative non-self-recognition' system[7,8]. Thus, conceptually, the S-RNase SI system operates in a very different way to the other SI systems.

In this review, we describe recent advances in our understanding of the SI systems of the Brassicaceae and the Papaveraceae. Although these systems utilise different modes of genetic control for SI and have completely different *S*-determinants, they share several similarities, including the site of inhibition — the stigma surface (Figure 1B,C). However, the mechanisms used to prevent self-fertilisation are quite different. In recent years, our knowledge of the network of signalling pathways and cellular mechanisms involved in regulating self-pollen rejection has improved considerably in these two SI systems. This provides us with information not only about the diverse ways in which plant cells recognise each other and activate SI rejection responses, but also about the intricate mechanisms that regulate the normal hydration of pollen grains and growth of compatible pollen tubes.

The cysteine-rich peptides specifying SI in the Brassicaceae and Papaveraceae

The identity and nature of the pistil and pollen *S*-determinants hold the key to how recognition is specified, and the *S*-determinants in both *Brassica* and *Papaver* function as receptor–ligand pairs. Intriguingly, though, they are reversed in their tissue location: the *Brassica* *S*-receptor kinase (SRK) localises to the plasma membrane of the stigmatic papilla cells, and the pollen coat contains the secreted SCR/SP11 ligand (Figure 1B), while the *Papaver* PrpS 'receptor' localises to the pollen plasma membrane and the stigmatic papilla cells secrete the ligand PrsS (Figure 1C).

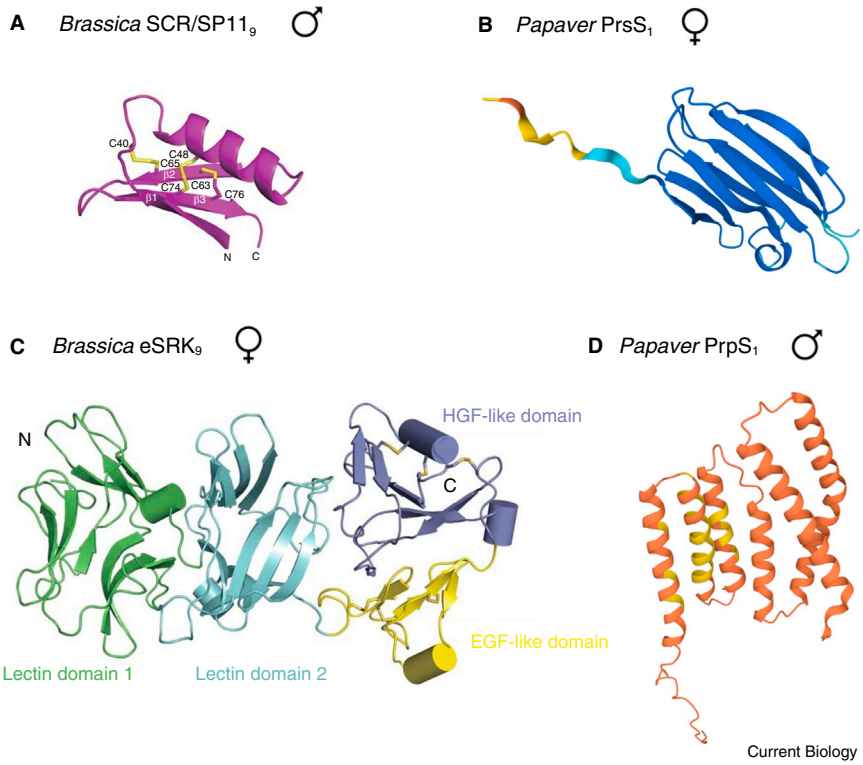

Figure 2. Structures and structural predictions of the *Brassica* and *Papaver* *S*-determinants.

(A) Structure of the *Brassica* male *S*-determinant, SCR/SP11$_9$[17]. SCR/SP11$_9$ has a defensin-like structure formed by four conserved disulphide bonds (three are shown in this structure). (B) Structural prediction of the female *S*-determinant, PrsS$_1$ (Uniprot Q40975), from *Papaver rhoeas*. Structural predictions were made using the EBI Alphafold structure database[26,27]. Colour code: dark blue, very high confidence prediction; pale blue, high confidence; yellow, low confidence; orange, very low confidence. (C) Structure of the extracellular domain (eSRK) from the female *S*-determinant, SRK$_9$, from *Brassica rapa*[17]. The SRK extracellular domain typically contains two lectin domains, followed by an EGF-like domain and an HGF-like (PAN-apple) domain. There are three hypervariable (HV) regions found in the lectin domain 2/EGF-like domain region that contribute to *S*-haplotype-specific binding between SCR/SP11 and eSRK[18]. (D) Structural prediction of the male *S*-determinant, PrpS$_1$ (Uniprot B3CJF9), from *Papaver rhoeas*. Structural predictions were made using the EBI Alphafold structure database[26,27]. Colour code: dark blue, very high confidence prediction; pale blue, high confidence; yellow, low confidence; orange, very low confidence. (A,C) Reprinted from Ma *et al.*[17], copyright 2016, with permission.

In the Brassicaceae, the identification of the pollen *S*-determinant, *SCR/SP11*, emerged from studies of small cysteine-rich pollen coat proteins (PCPs) that suggested that a PCP might act as the peptide signal for SRK activation[9,10]. Searches of the *S*-locus genomic region for a polymorphic *PCP* gene led to the identification of *SCR/SP11*[11] and the demonstration that this is indeed the pollen *S*-determinant[12]. SCR/SP11 was then shown to function as the ligand for SRK, as it binds with high affinity to the SRK extracellular domain to stimulate autophosphorylation of the intracellular kinase domain[13–15]. SCR/SP11 is a small (~9 kDa), cysteine-rich member of the defensin superfamily. The overall structure has been solved for several SCR/SP11 proteins, and they typically form a plant defensin-like structure with eight conserved cysteines forming four disulphide bonds (Figure 2A)[16–18]. Outside of the conserved cysteines, the SCR/SP11 amino acid sequences are quite diverse, contributing to the *S*-haplotype-specific binding of SCR/SP11 to its cognate SRK[16,18].

In *Papaver*, the female *S*-determinant, PrsS, is a small (~15 kDa) protein secreted by the stigmatic papilla cells[19]. Sequence information for four *PrsS* alleles in *P. rhoeas* revealed that the primary amino-acid sequence of the proteins encoded by PrsS is highly polymorphic (40–46% divergence between alleles[20]), but all of these proteins have a highly conserved predicted secondary structure comprising several β-strands separated by hydrophilic loops. No obvious hypervariable regions exist, but site-directed mutagenesis revealed that sites in hydrophilic loops 2 and 6 are essential for biological activity[21]. More recently, as many as 87 unique putative stigmatic *S*-allele sequences have been identified in various species within the Papaveraceae[22]. Seed set data from crosses showed strong correlation between genotype and SI phenotype, suggesting that the *S*-allele sequences are functional pistil *S*-alleles or paralogues of the *S*-locus[22]. When they were identified, PrsS proteins had no clear homologues in the databases, but they were subsequently found to be members of a large protein family named SPH (*S*-protein homologue)[23]. In *Arabidopsis thaliana*, the SPH family has at least 75 members[24]: the structure of one of these, SPH15, has recently been solved. SPH15 has a β-sandwich structure, with between eight and nine β-sheets in a topology distinct from that found in most other proteins to date. Several unrelated proteins have domains with the same topology, including the membrane-binding domain of the bacterial proteins pneumolysin and perfringolysin, although there is no discernible sequence similarity[24]. Intriguingly, these proteins are toxins that form oligomeric rings, comprising large transmembrane β-barrels, that form pores in eukaryotic membranes[25]; if PrsS proteins also form pores, this suggests that they might play a direct role in ion influx during interaction with PrpS. The predicted topology of PrsS proteins is the same as that of SPH15[24]; see Figure 2B for a structural prediction for PrsS$_1$ using Alphafold[26,27].

Although they are quite different proteins, both PrsS and SCR/SP11 fall into the large family of cysteine-rich peptides (CRPs). CRPs are small, secreted peptides/proteins of up to 150 amino-acid residues with an amino-terminal signal peptide and usually an even number of at least four conserved cysteine residues. It has been estimated that at least 825 genes encode CRPs in *Arabidopsis*[28]. CRPs bind a variety of receptors (many utilising co-receptors) via diverse binding modes[29,30], indicating divergent signalling strategies. Many CRPs are antimicrobial peptides involved in defence, preventing pathogen growth.

Defensins, which function in plant innate immunity, are thought to be the most ancient of CRPs[28]. However, numerous CRPs are expressed in reproductive tissues[31–33] and are thought to function in pivotal cell–cell interactions during various pollination events. These include pollen coat proteins (PCPs), the *Brassica* SCR/SP11 *S*-determinant, rapid alkanisation factor (RALF) proteins, LURE proteins and the *Papaver* PrsS proteins[31,34,35]. It has previously been suggested that SI may have evolved from defence pathways[36,37], and the overall similarities — including shared cell–cell recognition functions, extensive polymorphism and evolution of new recognition specificities, and bioactivity in regulating growth through triggering of signalling networks — are tantalising. Although several CRPs have a role in cellular communication during reproduction, many CRP peptide–receptor pairs remain to be identified.

The contrasting *S*-determinant 'receptors' in the Brassicaceae and Papaveraceae

While the ligands of the Brassicaceae and Papaveraceae SI systems are both CRPs, the receptors with which they interact are quite dissimilar. The *Brassica* female *S*-determinant, SRK, responsible for specifying rejection of self-pollen, localises to the plasma membrane of the stigma papilla cells[38] and is a receptor serine/threonine kinase related to a large family of plant receptor-like kinases (RLKs)[39,40]. The extracellular domain of SRK (eSRK) has several conserved domains found in this subfamily of RLKs, and three hypervariable regions that contribute to *S*-haplotype-specific binding to its cognate SCR/SP11 (Figure 2C). Structural studies on the *Brassica* SRK–SCR/SP11 complex have revealed new information about the molecular interactions[17]. Ligand binding of SCR/SP11 to SRK triggers homodimerisation of eSRK, leading to the formation of a heterotetrameric complex composed of two molecules of SCR/SP11 and two molecules of SRK; this involves binding of SCR/SP11 to the three extracellular hypervariable regions in eSRK[17]. Simulations predict that the binding free energies between SRK and SCR/SP11 are more stable between cognate *S*-haplotypes than between non-cognate *S*-haplotypes[18]. The differences between multiple contact regions in SRK and SCR/SP11 also contribute to the *S*-haplotype-specific interactions; thus, these studies have revealed amino-acid residues critical for guiding *S*-haplotype-specific receptor–ligand interactions[18].

The *Papaver* male *S*-determinant, PrpS, is a small (~20 kDa) transmembrane protein found in the plasma membrane. It has a predicted extracellular loop region of ~35 amino acids; peptides from this region bind PrsS in an *S*-specific manner[41], suggesting that it functions as a receptor. However, PrpS is something of an enigma, as extensive searches of sequence databases have failed to identify any orthologues of *PrpS* genes. Thus, it is clearly not a conventional 'classic' receptor, and it could be argued that it should not be called a receptor. However, for the purposes of this review, we will regard it as such, as interaction with the cognate PrsS triggers an intracellular signalling network involving classical signalling components, resulting in highly specific biological responses in incompatible pollen (see section titled Unravelling mechanisms downstream of S-determinant interaction in the Papaveraceae). Moreover, the expression of *PrpS* in self-compatible *A. thaliana* pollen and subsequent addition of recombinant cognate PrsS results in downstream SI events[42], demonstrating that PrpS alone is required for transducing the required SI signalling network in incompatible pollen. However, as the PrpS sequence contains no kinase or other identifiable domains, it is clearly not a RLK or a receptor-like protein, which are the usual plant receptors located at the plasma membrane. This raises a question about the origins and evolution of SI, as well as about how PrpS transduces a signal after ligand binding.

Sequence information predicts that PrpS encodes a highly hydrophobic protein with several transmembrane domains, but the exact topology is not yet known (Figure 2D shows a structure prediction for PrpS using Alphafold[26,27]). Intriguingly, the *Drosophila* protein Flower, which is involved in presynaptic vesicle endocytosis[43], is a 'topological homologue' of PrpS. Although both Flower and PrpS share very little primary sequence homology, they both have several conserved acidic residues in a proposed transmembrane domain, a characteristic of voltage-gated Ca^{2+} channels. Flower forms a homomultimeric complex and functions as a Ca^{2+}-permeable channel. Intriguingly, PrpS has three aspartic acids and three glutamic acids conserved across the three identified PrpS alleles[41,44]; several are close to putative predicted transmembrane domains and are candidate amino-acid residues for generating pore/channel selectivity. Thus, PrpS might multimerise to form a channel. As SI in *Papaver* triggers Ca^{2+} signalling and influx (see section titled Unravelling mechanisms downstream of S-determinant interaction in the Papaveraceae), this is an interesting proposition to investigate in the future.

Unravelling mechanisms downstream of *S*-determinant interaction in the Brassicaceae

When pollen is recognised as compatible, the stigmatic papilla cells release water for hydration of the desiccated pollen grain. Typically, within 30 minutes of pollination, a compatible Brassicaceae pollen grain will have hydrated and germinated, and the emerging pollen tube will have started to grow through the stigmatic papilla cell wall towards the base of the cell. The growing pollen tube will then enter the reproductive tract, following cues towards an unfertilised ovule for sperm cell release and double fertilisation (reviewed in[45,46]). The Brassicaceae SI response is very rapid and disrupts the early stages of pollen hydration and germination; any emerging SI pollen tubes fail to grow into the stigmatic papilla cell wall[47] (reviewed in[48,49]).

This rapid SI response is initiated within the stigmatic papilla cells when there is an *S*-haplotype match between the pollen-producing anther and the pistil, and SCR/SP11 from the surface pollen coat binds with high affinity to SRK in the plasma membrane of the stigmatic papilla cell to stimulate SRK autophosphorylation[13–15]. Specific to *Brassica* genomes is a third *S*-locus-linked polymorphic gene that encodes the *S*-locus glycoprotein (SLG), a secreted glycoprotein with homology to the SRK extracellular domain[50]. In transgenic *Brassica* studies, however, SLG was found not to be essential for SI but might strengthen the SI response[40,51]. The downstream signalling pathway has been best characterised in *Brassica* and *Arabidopsis* species, and, while there are common components in these pathways, there also appear to be some genus-specific distinct elements that influence how SI pollen is rejected (Figure 3).

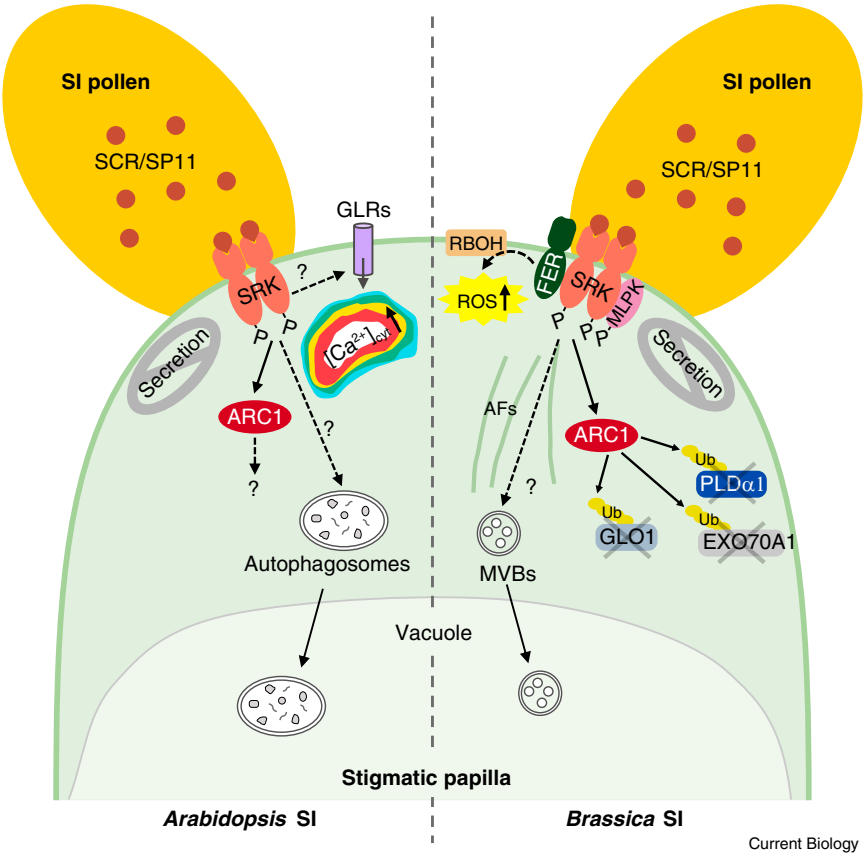

Figure 3. Components of the Brassicaceae SI pathway identified in *Arabidopsis* and *Brassica* species.

Following pollination with an *S*-haplotype-matched interaction (SI pollen), the pollen signalling peptide SCR/SP11 binds and activates the receptor kinase SRK in the plasma membrane of the stigmatic papilla. SRK autophosphorylation is followed by the activation of downstream cellular responses to reject the SI pollen. As illustrated in this model, studies on the downstream SI signalling events in *Arabidopsis* (left) and *Brassica* (right) have revealed a number of components, including some that appear to be unique to each genus. In general, these SI responses are designed to target compatibility factors and cellular events that would normally be needed for compatible pollen acceptance; for example, the disruption of secretion in the stigmatic papilla is a shared SI target in both pathways. In *Arabidopsis*, there is a rapid increase in cytosolic free calcium ($[Ca^{2+}]_{cyt}$), which is proposed to occur by Ca^{2+} influx through glutamate receptor-like channels (GLRs) and a rapid activation of autophagy. However, how the GLRs and autophagy are activated is not known. Both the rapid rise in $[Ca^{2+}]_{cyt}$ and the activation of autophagy are predicted to disrupt cellular responses needed for compatible pollen acceptance, leading to SI pollen rejection. In *Brassica*, the ARC1 E3 ligase (activated by SRK) has been implicated in targeting three compatibility factors: glyoxalase 1 (GLO1), the EXO70A1 exocyst subunit and phospholipase D α1 (PLDα1). In addition, *Brassica* SRK binds to the FERONIA (FER) receptor kinase, which activates a second intracellular pathway leading to the activation of plasma membrane-localized NADPH oxidases (RBOHs) to increase the production of reactive oxygen species (ROS) to inhibitory levels in the stigma. The disruption of actin filaments (AFs) and the rerouting of multivesicular bodies (MVBs) to the vacuole has also been observed in *Brassica*. Ultimately, all of these events would disrupt secretion and cellular homeostasis, leading to SI pollen rejection.

Recently, a second receptor kinase, FERONIA (FER), has been implicated in *B. rapa* SI where it is proposed to activate NADPH oxidases (RBOHs) to increase the accumulation of reactive oxygen species (ROS) to inhibitory levels, causing the rejection of SI pollen[52,53]. The suppression of *FER* expression in the stigma by antisense oligodeoxyribonucleotides led to a reduction in ROS accumulation and a breakdown in the rejection of SI pollen[52]. FER was also found to bind to SRK, and the addition of the corresponding *S*-haplotype SCR/SP11 ligand enhanced this interaction[53] (Figure 3). FER is a ubiquitous receptor kinase implicated in a wide range of cellular pathways, including the activation of NADPH oxidases for ROS production[54]. Three additional *Brassica* proteins have been directly linked with SRK and SI: thioredoxin h-like (THL) 1/2[55], *M* locus protein kinase (MLPK)[56], and ARM repeat containing-1 (ARC1)[57]. *Brassica* THL1/2 interact with the SRK kinase domain and inhibit basal SRK activity prior to the arrival of SI pollen[9,55,58]. MLPK was discovered when a mutant version of the gene was identified as the cause of loss of SI in a naturally occurring self-compatible *B. rapa* variety. MLPK belongs to the receptor-like cytoplasmic kinase (RLCK) family and, as is typical for this family, lacks an extracellular domain but localises to the plasma membrane where it can interact with SRK[56,59]. MLPK's importance as a positive regulator of SI was confirmed when *MLPK* homologues were mutated in *B. napus* using CRISPR/Cas9 and found to cause a complete loss of SI[60]. Both SRK and MLPK can phosphorylate the third SRK-interacting protein, ARC1[57,61] (Figure 3). *Brassica* ARC1 is a plant U-box E3 ubiquitin ligase[62] and is another positive regulator of SI. Its requirement was demonstrated through the partial loss of SI detected in ARC1-antisense knockdown transgenic *B. napus* lines[63] and the complete loss of SI in *B. napus ARC1* knockout mutants generated by CRISPR/Cas9[64]. One important feature of SRK and ARC1 is that they are solely expressed in the stigma, as this is where the SI pathway functions (Figure 3). Interestingly, MLPK has a stigma-specific isoform that localises to the plasma membrane through an amino-terminal hydrophobic domain, and this localisation is essential for MLPK to function in the SI pathway[59].

ARC1 orthologues are present in self-incompatible *Arabidopsis* species, and a requirement for *ARC1* in SI was revealed when the RNAi-mediated knockdown of *ARC1* in transgenic *Arabidopsis lyrata* led to a partial breakdown of SI[65]. However, the role of ARC1 is less clear from studies of transgenic *A. thaliana* SI lines. During the evolution of *A. thaliana* to a selfing species, inactivating mutations were acquired in the *SCR/SP11*, *SRK* and *ARC1* genes (reviewed in[48,66]). SI can be re-established in *A. thaliana* by transforming these SI genes from closely related self-incompatible species, but *ARC1* is not always required[67–70]. Studies

reported that the addition of *SCR/SP11* and *SRK* transgenes to the *A. thaliana* Col-0 accession failed to produce an SI phenotype yet were successful in the C24 accession[67,68,70]. However, the addition of an *ARC1* transgene to the transgenic *SCR/SP11-SRK A. thaliana* Col-0 plants led to an SI phenotype[69,71]. To add to the complexity, the *A. thaliana* Col-0 and C24 genomes still carry remnants of the *SRK* gene (pseudo *SRK*), which were discovered to have inverted repeats producing small RNAs (sRNAs)[72]. These sRNAs were able to suppress *SRK* transgene expression, but the diverging results between the Col-0 and C24 accessions in the transgenic studies appeared to result from the location of these SRK inverted repeats. For Col-0, the SRK inverted repeats were in a conserved kinase domain region, allowing the targeting of different *SRK* alleles, while the C24 SRK inverted repeats were in the more divergent extracellular domain and did not target the *SRK* transgenes that had been tested[72]. Somehow, adding an *ARC1* transgene in the Col-0 accession was able to compensate for the potential gene-silencing effect of the pseudo *SRK* sRNAs[69,71]. Overall, these findings suggest that there is redundancy in the SI pathway and that other branches in the pathway are still functioning to reject SI pollen in the transgenic *A. thaliana* SI lines (Figure 3). Redundancy in signalling is not unusual and is in fact a common theme in the networks that regulate plant–pathogen interactions[73,74].

Another interesting observation emerging from the transgenic *A. thaliana* studies was that the re-establishment of SI in *A. thaliana* was only successful with *SCR/SP11* and *SRK* from closely related SI species (*A. lyrata*, *A. halleri*, and *Capsella grandiflora*) and not from the more distantly related *Brassica* species[71,75]. In a recent study, some success was achieved when *B. rapa*/*A. lyrata SRK* chimeras were transformed, along with the corresponding *B. rapa SCR/SP11* alleles[75]. The chimeric transgenes contained *B. rapa* SRK extracellular and transmembrane domains fused to an *A. lyrata* SRK cytosolic kinase domain. This combination would be predicted to allow the *S*-haplotype specific recognition of the *B. rapa* SCR/SP11 by the *B. rapa* SRK extracellular domain to stimulate autophosphorylation of the *A. lyrata* SRK cytosolic kinase domain. Chimeric *SRK*s from five *Brassica S*-haplotypes were tested, and two successfully produced SI phenotypes in the transgenic *A. thaliana* lines[75], suggesting that sequence divergence of the *Brassica* SRK cytosolic kinase domain impacts interactions with downstream *Arabidopsis* signalling proteins. Ultimately, these results may indicate that the downstream SI pathway is not fully conserved across the Brassicaceae family and that there may be tribe/genus-specific differences in the cellular responses. While a number of components have been identified in the Brassicaceae SI pathway, so far there has only been partial overlap between the *Brassica* and *Arabidopsis* studies (Figure 3).

Since SI pollen also carries signals for compatible pollen recognition, the activation of the SI pathway essentially activates responses in the stigmatic papilla cells that shut down the compatible pollen response pathway (Figure 3). SRK activation is proposed to lead to ARC1 activation. ARC1, as an E3 ubiquitin ligase, will target proteins for ubiquitination and proteolysis (reviewed in Abhinandan *et al.*[48]). In *B. napus*, three different substrates have been identified as targets of ARC1: glyoxalase 1 (GLO1)[76], the EXO70A1 exocyst subunit[77], and phospholipase D α1 (PLDα1)[78] (Figure 3). A common theme of these diverse *B. napus* ARC1 targets is that they are factors required in the stigma to promote compatible pollen–stigma interactions. GLO1 is a detoxifying enzyme for methylglyoxal (MG), a by-product of glycolysis. By reducing MG levels, the cytotoxic effects of MG are avoided, establishing conditions in the stigma for accepting compatible pollen. With SI activated, the degradation of *B. napus* GLO1 would cause MG levels to rise, leading to MG modifications of proteins that will disrupt cellular functions to prevent compatible pollen responses[76]. EXO70A1 is a subunit of the exocyst, a complex of eight proteins that acts as a tether for vesicles at the plasma membrane during exocytosis. An *Arabidopsis* MAP kinase cascade was found to be responsible for phosphorylating EXO70A1, causing it to relocalise to the plasma membrane, thereby setting up the stigmatic papillae to be receptive to compatible pollen[79]. All the *Arabidopsis* exocyst subunit genes have been shown to be required in the stigma to support compatible pollen–stigma interactions[77,80]. By targeting *B. napus* EXO70A1 for ubiquitination and proteolysis in the SI response, secretion is disrupted in the stigmatic papilla, which in turn causes SI pollen rejection[77,80,81] (Figure 3). *B. napus* PLDα1 is also proposed to be involved in vesicle trafficking with compatible pollinations and targeted by ARC1 in the SI pathway[78]. PLDs hydrolyse phospholipids to produce phosphatidic acid, an activity linked to different membrane functions including vesicle trafficking and membrane fusion[82]. With both EXO70A1 and PLDα1 connected to vesicle trafficking, this points to the regulation of secretion as a key intersection point for the compatible pollen and SI pathways[77] (Figure 3). Transmission electron microscopy (TEM) studies of *Arabidopsis* pollinations have revealed evidence in support of this model[69,81]. Curiously, TEM studies of *Brassica* stigmatic papillae showed that, with compatible pollen, multivesicular bodies (MVBs) fuse to the plasma membrane of stigmatic papilla cells to release vesicles into the cell wall, whereas SI pollinations resulted in MVBs localising within the vacuole[49,78,81,83] (reviewed in Goring[84]).

A dynamic actin cytoskeleton is crucial for mediating vesicle trafficking[85]. Alterations to the configuration of the stigmatic papilla actin network were observed in *B. rapa* pollinations, where actin bundles were oriented towards the site of the compatible pollen grain, but disrupted in the region of SI pollen contact[86]. Similarly, studies of transgenic *A. thaliana* stigmatic papillae revealed 'focalised' actin bundles adjacent to compatible pollen but absent with SI pollen[47]. Ca^{2+} signalling is responsible for many alterations in cellular actin reconfiguration/reorganisation[87,88]. Rapid changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) have been observed in transgenic *A. thaliana* stigmatic papillae following both compatible and SI pollinations[70,89] (Figure 3), the key difference being the size of these fluxes. Much larger $[Ca^{2+}]_{cyt}$ fluxes were observed in the stigmatic papillae with SI pollen, with glutamate receptor-like channels being implicated in this response[70]. Although the mechanism of activation of these channels and the outcome of this activation are not known, the increases in $[Ca^{2+}]_{cyt}$ could potentially trigger disruption of the actin cytoskeleton (reviewed in[84,90]). Small $[Ca^{2+}]_{cyt}$ fluxes were observed with compatible pollinations and these could be associated with vesicle secretion[70,89]. Finally, studies on SI in *A. lyrata* and transgenic *A. thaliana* SI lines have uncovered a role for autophagy during the *Arabidopsis* SI response that could be linked again to the disruption of secretion[69,81,91]

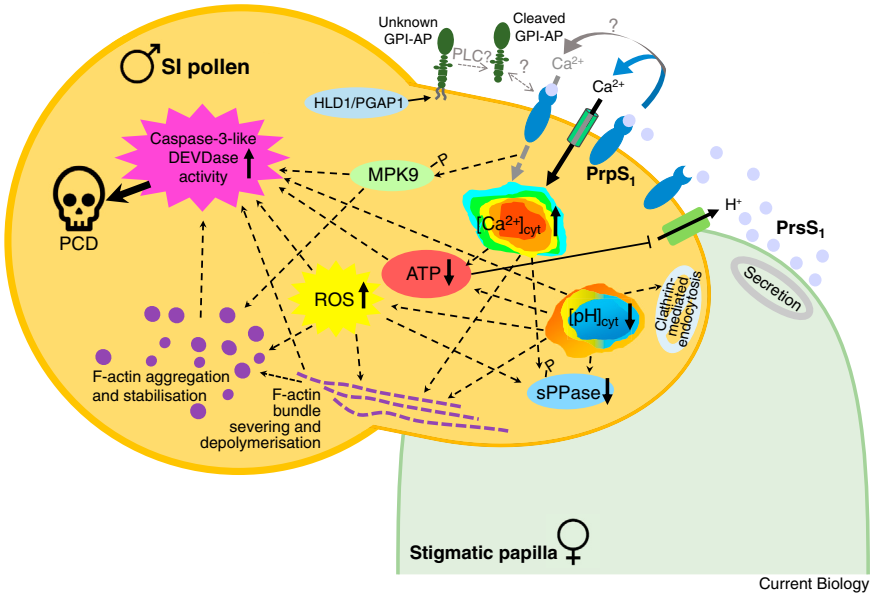

Figure 4. Components of the *Papaver* SI pathway.

A pollen grain germinating on a stigmatic papilla undergoes an SI response if the *S*-haplotypes match (here, $PrsS_1$ with $PrpS_1$). Note that, in contrast to the Brassicaceae, the rejection response takes place in the pollen. PrpS, the pollen *S*-determinant, is a transmembrane protein that acts as a receptor for the female *S*-determinant, PrsS, which is a ligand secreted by the stigma. Identification of a requirement for the glycosylphosphatidylinositol (GPI)-inositol deacylase HLD1/PGAP1 demonstrates that inositol deacylation, required for maturation of GPI-anchored proteins (GPI-APs), is critical for the SI response. It is proposed that, as well as the *S*-specific interaction of PrsS with plasma-membrane-localised PrpS, SI induction requires interaction (direct or indirect) of PrpS–PrsS with (as yet unknown and possibly cleaved) GPI-APs, which may act as co- or accessory receptors. After a cognate interaction, the SI pathway is rapidly activated to reject the incompatible pollen, first by inhibiting tip growth and then by initiating programmed cell death (PCD) to ensure prevention of fertilisation. Cognate secreted stigma PrsS interacts with PrpS, located at the pollen plasma membrane, triggering a signalling network involving rapid Ca^{2+} influx (though the nature of the channel involved is not yet known) and increases in cytosolic free calcium ($[Ca^{2+}]_{cyt}$). Increases in reactive oxygen species (ROS), dramatic depletion of ATP and acidification of cytosolic pH ($[pH]_{cyt}$) occur within 10 minutes of SI induction. ATP depletion is likely to cause H^+-ATPase pump inactivation. Activity of a soluble inorganic pyrophosphatase (sPPase) is inhibited, resulting in inhibition of cellular biosynthesis. Increases in $[Ca^{2+}]_{cyt}$ and ROS and a reduction in $[pH]_{cyt}$ also trigger alterations to the actin cytoskeleton, which rapidly undergoes severing and depolymerisation. Clathrin-mediated endocytosis is also rapidly inhibited by SI. These very early events all contribute to the rapid inhibition of pollen tube tip growth within 1–2 minutes of SI initiation. Increases in $[Ca^{2+}]_{cyt}$ and ROS and a reduction in $[pH]_{cyt}$ signal to several downstream targets to trigger PCD. An MPK9 homologue is activated by phosphorylation and plays a central role in SI, being required for SI-induced actin alterations and PCD. The actin fragments subsequently aggregate into distinctive, highly stable actin foci that can trigger PCD. The massive (but incomplete) ATP depletion creates a cellular energy crisis that not only rapidly inhibits pollen tube growth, but also triggers a drop in $[pH]_{cyt}$. This plays a key role in several pivotal SI-induced events, including actin remodelling. Cytosolic acidification is critical for initiation of PCD because the caspase-3-like DEVDase enzyme is inactive at normal $[pH]_{cyt}$. Activation of this enzyme, several hours after the initiation of the signalling network, seals the fate of the incompatible pollen, with a commitment to cell suicide ensuring that the inhibited SI pollen tubes do not achieve fertilisation.

(Figure 3). The observed MVBs in the *B. napus* vacuoles in response to SI pollinations may also be a sign of autophagy, but this has not been confirmed[81].

In summary, the SI responses observed in the Brassicaceae are designed to target compatibility factors and cellular events in the stigmatic papilla that would normally be needed for compatible pollen acceptance. The SI signalling network triggers signals that affect several targets that regulate secretion and homeostasis in the stigmatic papilla, with the consequence of preventing SI pollen hydration and/or germination, thereby resulting in pollen rejection (Figure 3).

Unravelling mechanisms downstream of *S*-determinant interaction in the Papaveraceae

The two *Papaver S*-determinants have been successfully transferred to *A. thaliana*, which is normally self-compatible, resulting in plants that were completely self-incompatible, with no seed set[92], and demonstrating that *PrsS* and *PrpS* can act as a functional synthetic *S*-locus. This suggested that, on the face of it, just these two *S*-determinants are required for SI. However, a recent study has uncovered a requirement for glycosylphosphatidylinositol-anchored proteins (GPI-APs) as an additional component of SI[93]. A study using the transgenic *A. thaliana* SI lines[92] showed that knockout of the *HLD1/PGAP1* gene, which encodes an orthologue of the mammalian GPI-inositol deacylase PGAP1, causes a complete loss of SI[93]. This finding implicates a critical role for the remodelling of GPI-APs and their cleavage and release from the plasma membrane by deacylation and suggests that GPI-anchored proteins play a key role in *Papaver* SI[93]. As some GPI-APs function as co-receptors, enhancing receptor–ligand interactions through association with partner RLKs and their CRP ligands[94,95], this implicates a critical role for accessory proteins in *Papaver* SI. Although PrpS is not an RLK, these data suggest that its activity or interaction with PrsS could potentially be regulated by a GPI-AP co-receptor.

Downstream of the haplotype-specific interaction of PrsS and PrpS, Ca^{2+} influx from the stimulation of a non-specific cation channel[96] leads to rapid, transient increases in $[Ca^{2+}]_{cyt}$ in incompatible pollen tubes. This triggers a Ca^{2+}-dependent signalling network that rapidly inhibits tip growth and later culminates in programmed cell death (PCD) in incompatible pollen[97,98]. This cell suicide system provides a neat, targeted way to reject and kill incompatible pollen grains to prevent self-fertilisation (Figure 4)[99]. Several components of this SI-PCD signalling network have been identified. A pollen-expressed MAPK9 homologue, PrMPK9-1, is phosphorylated within a few minutes and its activation triggers upregulation of caspase 3-like DEVDase activity[100,101].

Within a few minutes of SI induction, increases in intracellular ROS are observed[102]. Although it is not yet known how ROS increases are induced in this system, the finding that H_2O_2 can trigger actin alterations in these pollen tubes implicates an

integral role for ROS in the SI-induced PCD (Figure 4). A number of targets for ROS have been identified. Irreversible protein oxidation is observed in incompatible pollen within 10 minutes of SI induction. Notably, enzymes involved in energy metabolism are a target[103], suggesting that SI promotes metabolic alterations. The SI-induced inhibition of the soluble inorganic pyrophosphatase (sPPase) Pr-p26.1 by SI-stimulated Ca^{2+}-dependent phosphorylation[104,105] supports this idea. As sPPase activity is crucial for cellular biosynthesis, inhibition of this activity could clearly contribute to the arrest of growth that is observed in SI. Intriguingly, phosphoregulation of key amino acids in p26.1 requires not only Ca^{2+} but also ROS (and pH; see later in this section) for the inhibition of the catalytic responsiveness of this PPase and it was proposed that this could act as a regulatory mechanism to attenuate metabolism[106]. Thus, Ca^{2+}-dependent phosphorylation and ROS play key roles that are pivotal to the regulation of SI in *Papaver* (Figure 4). ROS also mediates alterations to the actin cytoskeleton[102,103], a distinctive feature of the SI response. Incompatible *Papaver* pollen display dramatic alterations in F-actin organisation within minutes, providing evidence that F-actin is a very early target for SI signals. SI triggers rapid actin depolymerisation and subsequent aggregation of F-actin into distinctive 'foci' in incompatible pollen[107]. Transgenic *A. thaliana* SI lines expressing the *Papaver* S-determinants[92] have provided an engineered 'poppydopsis' system that allows the use of genetic approaches that are not possible in *Papaver*[108], including observation of live-cell actin dynamics[108,109]. This has yielded evidence for extensive F-actin severing during early SI and has revealed that subsequent formation of the SI-induced F-actin foci predominantly occurs through aggregation of small fragments of F-actin bundles[109]. The actin cytoskeleton is a complex dynamic network that undergoes rapid assembly and disassembly, and actin organisation plays a critical role in regulating pollen tube growth[110,111]. Actin depolymerisation is known to rapidly inhibit tip growth, so this is almost certainly a consequence of Ca^{2+}-stimulated actin depolymerisation. In animal cells and yeast, actin stabilisation has a pivotal role in apoptosis[112]. Actin depolymerising/stabilising drugs both resulted in the activation of caspase-3-like DEVDase activity in *Papaver* pollen tubes[113], establishing the importance of actin dynamics/remodelling in mediating SI-PCD in pollen tubes (Figure 4).

Pollen tube growth is a process that consumes high levels of ATP[114]. It was recently shown that SI triggers rapid and significant (though not total) ATP depletion in incompatible pollen tubes. This is likely to inhibit pollen tube growth (as a consequence of altered cellular energy metabolism) and also to be important for SI upstream of PCD[109]. As the Ca^{2+} ionophore A23187 also triggered ATP depletion, this suggests that Ca^{2+} influx during SI plays a role in triggering reduction of ATP levels. Decreases in ATP synthesis are usually caused by mitochondrial defects, which can be triggered by ROS. As SI in *Papaver* induced the release of a key marker of PCD, cytochrome c[98], mitochondria are thought to play a role in ATP depletion upstream of PCD. Another distinctive feature of SI is the rapid and dramatic acidification of the cytosol of incompatible pollen tubes. The drop in intracellular pH is very extreme, reaching pH6.4 within 10 minutes and stabilising at pH5.5 at 60 minutes in *Papaver* pollen tubes[115]. Remarkably, the pollen tubes are still alive at this stage, suggesting that this is an active, controlled process. Artificial ATP depletion in both *Papaver* and 'poppydopsis' pollen tubes triggered acidosis similar to that triggered by SI[109], implying that ATP plays a critical role in regulating this phenomenon. ATP depletion is likely to cause plasma membrane H^+-ATPase pump inactivation, given that ATP is required for H^+ efflux, and failure to export H^+ could cause further acidification[109,116] (Figure 4).

Cytosolic acidification has a central role in SI because it affects several critical cellular components (Figure 4). The activity of the sPPase p26.1 is inhibited by low intracellular pH as well as by Ca^{2+} and ROS[106,115]; the consequent inhibition of biosynthesis results in the arrest of tip growth. Clathrin-mediated endocytosis (CME) is required for pollen tube growth[117]. Within a few minutes of SI induction, recruitment of TPLATE — one of the eight subunits of the endocytic TPLATE complex[118] — at the pollen tube plasma membrane was reduced, revealing that CME is significantly inhibited by SI[108], but the exact mechanisms are currently unclear. Although CME is an energy-dependent process and ATP depletion significantly reduces CME dynamics in plant cells, cytoplasmic acidification triggers inhibition of CME in pollen tubes; low intracellular pH has been shown to be the primary cause for CME inhibition in plant cells[119]. Another target is the actin cytoskeleton. The formation of actin foci is stimulated by artificially lowering the intracellular pH and prevention of acidification in SI-induced pollen tubes blocked foci formation[115]. This illustrates the functional importance of acidification in the SI response and reveals a role for actin foci in this pathway. A pivotal function for cytosolic acidification is activation of the caspase-3-like DEVDase enzyme involved in executing PCD in incompatible pollen tubes. This enzyme is completely inactive at normal cytosolic pH (~pH6.8) and requires an optimal pH of 5.0 *in vitro*[97,115]. Thus, the acidification of the pollen cytosol is critical in activating this enzyme for the execution/progression of PCD in *Papaver* pollen. Although it is not known how cytosolic acidification is achieved, it clearly triggers cellular events responsible for the PCD of incompatible *Papaver* pollen tubes.

In summary, *Papaver* SI involves a complex signal transduction network involving Ca^{2+}, H^+ and ROS that modifies several intracellular targets in incompatible pollen. This not only results in rapid arrest of pollen tube tip growth, but also triggers an integrated network of events that leads to PCD to ensure that incompatible pollen cannot recover and is permanently rejected (Figure 4).

Commonalities in cellular events downstream of PrpS and SRK activation

Both the *Papaver* and Brassicaceae SI systems involve complex cellular responses downstream of pollen–stigma interactions for SI pollen rejection. Though the sites of these responses differ, there is an overall common theme of initiation of destructive pathways to stop key processes required for compatible pollen–pistil interactions. The SI responses occur in the *Papaver* pollen tube to stop pollen tube growth and in the stigmatic papilla in the Brassicaceae to stop cellular responses needed for pollen hydration and germination. In *Papaver* SI, Ca^{2+} signalling triggers actin remodelling in incompatible pollen. A similar scenario may occur in SI in the Brassicaceae, as large increases in $[Ca^{2+}]_{cyt}$ and actin reorganisation have been observed in *Arabidopsis*

stigmatic papillae and *Brassica* stigmatic papillae, respectively. These alterations to the actin cytoskeleton would impact vesicle trafficking; essentially, the disruption of secretion in the stigmatic papilla of Brassicaceae and CME in pollen tubes in *Papaver* are predicted to affect critical endomembrane dynamics required for compatible pollen and represent additional features in the SI-induced signalling pathways that are common to both systems. Increased ROS production is another common element in both SI systems, implicating targeting of metabolic processes as observed in *Papaver* SI. These SI systems also target essential enzymes for cellular homeostasis, such as the *Papaver* sPPase and *Brassica* GLO1. The end point of *Papaver* SI is the triggering of PCD in incompatible pollen; a potentially related process, autophagy, has been observed in stigmatic papillae for *Arabidopsis* SI, although this autophagic response has not been linked to PCD. Thus, there are several similar processes triggered by SI in these two SI systems, despite them being specified quite differently at a genetic level.

What is striking about both SI systems is that multiple pathways are initiated for SI pollen rejection to essentially shut down the *Papaver* pollen and the Brassicaceae stigmatic papillae in an incompatible situation (Figures 3 and 4). As more steps are uncovered in these SI pathways, additional commonalities are likely to emerge. For example, the involvement of FER in the *Brassica* SI pathway will likely lead to the identification of roles for other FER-associated signaling components such as RALF signalling peptides and the GPI-APs LORELEI (LRE) and LORELEI-LIKE (LLGs) LLG1/2/3 in ROS signalling. Recently, GPI-APs (yet to be identified) have been implicated in *Papaver* SI, raising the question of whether accessory proteins/co-receptors and perhaps associated RLKs will also be discovered as part of this pathway. Ultimately, uncovering this network of interacting components will reveal the elements that are crucial for the regulation of normal pollen tube growth.

Potential translational applications of SI

SI has been used by plant breeders for decades in the production of F1 hybrids. Knowledge about the molecular genetics of SI systems gathered over the last few decades suggests that this has the potential to be utilised for the benefit of food security, which is partly why much research on SI has been carried out in crop-related species. For example, knowledge of the *S*-genes regulating SI has led to the development of more precise genotyping methods to identify cross-compatible interactions for generating F1 hybrids in Brassicaceae crops (e.g. *Brassica* and *Raphanus* species) using the SI system or in combination with other hybrid breeding systems[120–123]. In addition, self-compatible crops can, in theory, be made SI by integrating foreign *S*-determinant transgenes. Conversely, SI crops could potentially be made self-compatible by using targeted gene silencing or CRISPR knock-out of SI-related factors (reviewed in Munoz-Sanz *et al.*[120]). This has been the goal for many SI researchers since the *S*-determinants were identified and cloned; however, it has been fraught with difficulties. For example, in the Brassicaceae, *SRK* and *SCR/SP11* have been transferred from closely related species *Arabidopsis lyrata* to the self-compatible species *A. thaliana* to make the latter SI[124], but this is effectively a restoration of components lost when SI species became self-compatible and wider trans-genera transfer of Brassicaceae *S*-determinants has been challenging[75]. Remarkably, though, the *Papaver* system has emerged as an SI system that appears to hold promise for such transfer. The successful transfer of the two *Papaver S*-determinants, *PrpS* and *PrsS*, to make *A. thaliana* (which diverged ~140 mya from the *Papaver* lineage) fully SI suggests that it may be now possible to introduce SI into widely diverged plant species and into crops. If this is possible, the *Papaver* system may be of practical use in the future[125]. As the *PrsS* and *PrpS* genes can also act as a synthetic *S*-locus in vegetative cells, with 'SI' leading to PCD[126], their ability to act independently of a reproductive context may provide further possibilities for applications. Moreover, the finding that knockout of the GPI-inositol deacylase *HLD1/PGAP1* restores self-compatibility in these synthetic SI plants[93] suggests that the ability to turn off SI in this system may also be a practical possibility in the future.

Conclusions and future challenges

We now know a great deal about the *S*-determinants as well as the components and mechanisms involved in mediating SI in *Brassica* and *Papaver*. These two SI systems both utilise a self-recognition system that clearly evolved independently, employing different *S*-determinants and mechanisms to prevent self-fertilisation. Comparisons reveal several similarities: both systems use highly polymorphic plasma-membrane-localised *S*-determinants (SRK and PrpS) that interact with highly polymorphic ligands (SCR/SP11 and PrsS). These small signalling ligands are both CRPs and trigger signalling networks downstream of their interaction with their cognate receptor. However, they utilise different types of receptors and downstream signalling pathways to elicit an SI response to inhibit incompatible (self) pollen and prevent self-seed-set. Although the outcomes and targets are quite different, these systems have in common the use of Ca^{2+} and ROS as signals and the targeting of metabolic processes and the cytoskeleton. It has been speculated that these SI systems may have evolved from self-/non-self-recognition systems used in plant defence against pathogens, such as the innate immune system, because elements of the SI recognition system and the downstream signalling networks share some similarities with these ancient polymorphic pathogen recognition/signalling systems. Elucidating the evolutionary origin of these SI systems will be an interesting challenge for the future.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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