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Founders' Review

Stop and go signals at the stigma–pollen interface of the Brassicaceae

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The key evolutionary innovation of the angiosperm closed carpel has resulted in the physical separation of the male gametophyte, which is housed in the pollen grain, and the female gametophyte, which is embedded in the ovule deep inside the ovary at the base of the pistil. Consequently, fertilization in angiosperms requires that the pollen tube and its cargo of haploid sperm cells travel through many layers of somatic pistil tissue. This journey starts at the receptive stigma surface to which pollen grains adhere and where they hydrate and germinate, continues with extracellular growth of the pollen tube through the stigma, style, and ovary, and culminates in entry of the pollen tube into the ovule, release of its sperm cells, and double fertilization. Throughout this journey, an intricate molecular dialogue between cells of the pistil and the pollen grain or pollen tube determines if pollination will ultimately result in fertilization and seed production or in interruption of pollen tube growth, failure of fertilization, and lack of fruit and seed (Johnson et al. 2019). In this way, pistil tissues function as selective gatekeepers that save female tissue resources and safeguard their ovules from unproductive fusions by discriminating against inappropriate (incompatible) pollen grains or tubes, be they derived from the same species (conspecific) or from different species (heterospecific), while allowing appropriate (compatible) pollen grains or tubes to effect fertilization.

The site in the pistil where discrimination against incompatible pollen occurs is dictated by the structural and biochemical characteristics of the stigma and the way in which pollen grains, which are released from anthers in a highly desiccated and metabolically inactive state, access water for hydration and subsequent metabolic activation. The surface properties of the stigma differ among plant families. In some families, the stigma is covered by an abundant exudate from which pollen grains can readily access water. As a result, these stigmas allow indiscriminate hydration and germination of conspecific and heterospecific pollen grains alike, and arrest of inappropriate pollen tubes must therefore occur within internal tissues of the pistil, most often in the style. By contrast, families such as the Brassicaceae, in which the stigma lacks abundant exudate, hydration of pollen grains can only occur upon release of water from the stigma. Thus, in these families, the pollen–stigma interaction is a highly regulated and selective process in which the stigma plays a critical role in determining the fate of pollen grains, with pollen hydration representing a major reproductive checkpoint.

Here, I present an overview of the complex molecular processes that underlie the cell-cell recognition events that take place at the pollen–stigma interface with a focus on the Brassicaceae (crucifers), a family in which these events have been extensively analyzed. After a description of the stigma–pollen interface, the review will highlight major results relating to (i) compatible pollinations for completion of the plant's life cycle, (ii) discrimination between self- and nonself-pollen for enforcing outbreeding and avoiding the deleterious effects of inbreeding, and (iii) discrimination between conspecific and heterospecific pollen for conservation of pistil resources and maintenance of species integrity.

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The stigma–pollen interface in crucifers

For successful pollination to occur in crucifers, contact with a stigma epidermal cell is a precondition for hydration, activation of a pollen grain, and establishment of a polarized growth pattern within the grain. Additionally, the resulting pollen tube gains access to internal pistil tissues by burrowing into the wall of the epidermal cell and emerging at its base before transitioning to an intercellular mode of growth in the subepidermal region of the stigma and transmitting tissue of the style and ovary (Fig. 1A). Thus, the cell–cell interactions at the stigma surface that lead to acceptance or arrest of pollen may be viewed as a one-on-one interaction between a pollen grain and a stigma epidermal cell, or more specifically, between molecules displayed at the surface of each of the two cells.

The epidermal cells of the stigma

The epidermal cells of the dry stigma are highly modified cells, called papillae, which are uniquely specialized for receipt of pollen and for discrimination between appropriate and inappropriate types of pollen (Fig. 1). In crucifers, the papillae are unicellular and uninucleate cells that grow into elongated finger-like or bottle-like cells that cover the stigma in a densely packed mound. The crucifer papilla plays a vital role as a source of factors required for the early steps of pollination and of signals that establish the normal basipetal growth of pollen tubes into underlying pistil tissues. Indeed, in wild-type plants, pollen grains will typically only adhere to, and germinate on, the surface of mature papillae but not on other epidermal surfaces of the plant, and adhesion and germination of pollen grains as well as directed pollen tube growth into underlying pistil tissues occur on mature stigmas with fully differentiated and elongated papillae but not on immature stigmas having rudimentary or undifferentiated papillae (Kandasamy et al. 1994).

The mature papilla has a pectocellulosic wall consisting of two layers, which may be a modification for pollen tube growth within the wall (Fig. 1A; Dickinson 1995). The papilla cell wall is covered by a protective cuticle, a lipophilic matrix of cutin and wax, which in turn is overlayed by a thin hydrated proteinaceous film called the pellicle (Dickinson 1995; Zinkl et al. 1999). The pellicle is of interest because it is the site of first contact between pollen grain and papilla, and its removal can prevent pollen tube penetration of papillae (Dickinson 1995). However, identifying the pellicle's constituents and pinpointing their specific function has been hampered by the unavailability of mutants lacking a stigma pellicle and the difficulty in isolating sufficient pellicle material for analysis.

The pollen grain

A pollen grain develops from a diploid microsporocyte in anther locules lined by the tapetum, a sporophytically derived secretory cell layer that serves as a source of compounds required for the normal development and maturation of microspores. Consequently, a pollen grain exhibits both gametophytically determined characteristics that result from the activity of genes within the grain's haploid genome and sporophytically determined characteristics controlled by molecules that are synthesized in the tapetum and are subsequently transferred to the pollen surface either by secretion from intact tapetal cells at early stages of microspore development or by release into the locule after degeneration of tapetal cells at later stages of microspore development. This dual gametophytic/sporophytic genetic control of pollen grain development is well exemplified by the assembly of the pollen wall.

The pollen wall is a complex multilayered structure consisting of 3 major layers which surround and protect the sperm cells (Wang and Dobritsa 2018). Adjoining the plasma membrane is the cellulosic intine layer containing various proteins such as enzymes and allergens which are under gametophytic control. External to the intine is the exine layer, whose main constituent is sporopollenin, a chemically inert mixed polymer consisting mainly of long-chain fatty acids and phenolics, the precursors of which are synthesized in the tapetum (Quilichini et al. 2015). The exine, which gives mechanical strength to the pollen grain, forms a rigid cover whose thickness is interrupted by apertures often used for pollen tube emergence. In many taxa, especially in insect-pollinated plants such as crucifers, the exine is highly sculptured and exhibits a species-specific reticulate pattern that might contribute to the species-specificity of pollination (Edlund et al. 2004). This pattern may be used for species identification even in 33-million-year-old flowers preserved in amber (Sadowski and Hofmann 2023). Cavities in the exine are filled with a largely tapetum-derived heterogenous viscous, sticky, and semi-solid matrix called pollen coat or tryphine. Together, the exine and pollen coat protect the male gametophyte from desiccation, mechanical injury, UV damage, and pathogen attack (Wang and Dobritsa 2018). They are also critical for successful reproduction. In insect-pollinated plants, the pollen coat contains volatile lipids that attract pollinators, and its viscosity aids in pollen dispersal by allowing the grains to adhere to each other and to pollinators (Edlund et al. 2004). And most relevant to the topic of this review, exine and pollen coat are critical for the earliest stages of the pollen-stigma interaction.

As the outermost component of the pollen wall, the pollen coat has long been considered the likely repository of molecules involved in pollen–stigma signaling, and as such, its constituents have received much attention. The pollen coat contains a large lipid fraction, wax esters, glycoconjugates, flavonoids, carotenoid pigments, and proteins (Edlund et al. 2004; Rejón et al. 2016). Unlike proteins of the stigma pellicle, pollen coat proteins are readily obtained by brief washes of pollen grains with organic solvents such as cyclohexane, followed by protein solubilization. Pollen coat proteomes include several major classes of proteins that may function in the pollen–stigma interaction, among which are hydrolytic enzymes thought to contribute to the modification of the papilla surface during pollen tube entry and a large diverse group of small (<10 kDa) cysteine-rich proteins (CRPs) (Rejón et al. 2016; Wang et al. 2023). These CRPs are of particular interest because they belong to a family of proteins that have been shown to function as signaling molecules in various plant

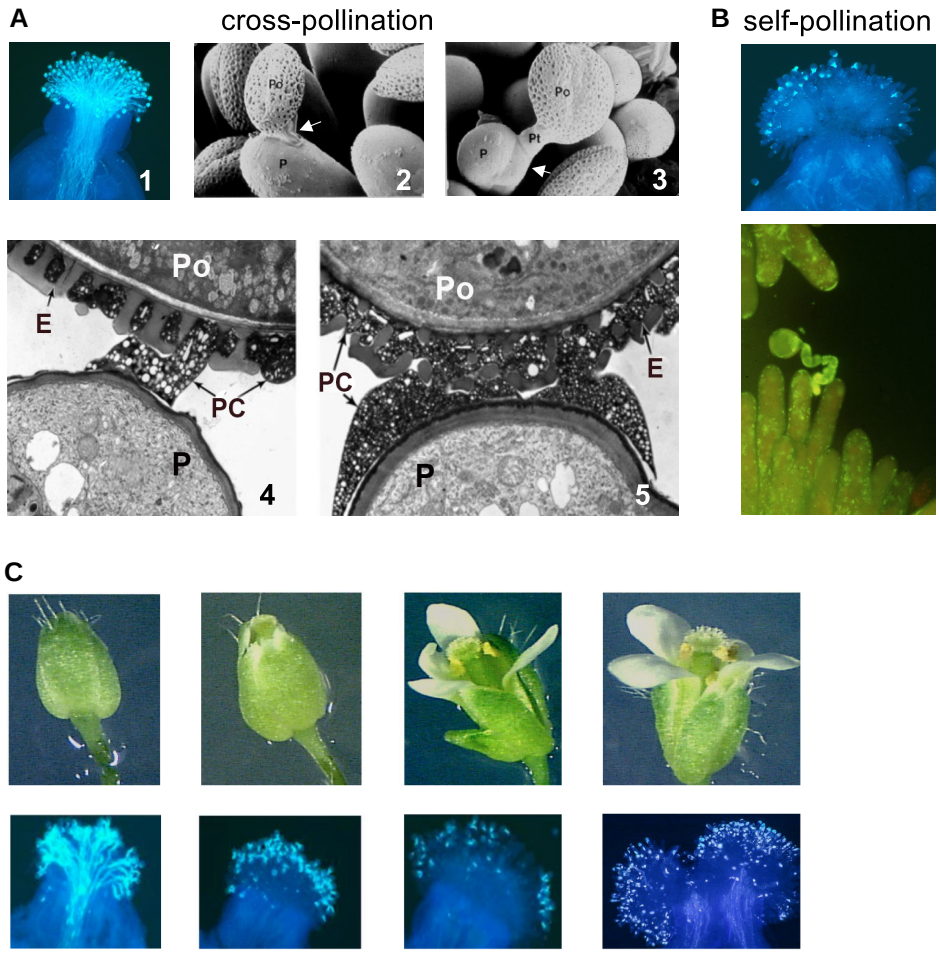

Figure 1. Cross- and self-pollination in crucifers. **A)** Pollination with cross-pollen. (1) Profuse pollen-tube growth as visualized by light microscopy. The images were generated by fixing pollinated stigmas, treating them with decolorized aniline blue which stains the callose in pollen tube walls, and visualization by UV fluorescence microcopy. (2 and 3) The pollen–papilla interaction as visualized by scanning electron microscopy. Arrows in (2) and (3) point to the attachment foot and to a pollen tube growing within the papilla cell wall, respectively. (4 and 5) Establishment of the attachment foot at the pollen–papilla interface as visualized by TEM. The images were taken at 5-min intervals during establishment of the attachment foot. **B)** Pollination with self-pollen. Most pollen grains are inhibited at the stigma surface due to failure of pollen hydration and germination (top), but in the few cases where the incompatible grain germinates, its tube fails to grow into the papilla cell wall (bottom). The light microscopy images were generated as in **A)**, except that a different filter was used for UV fluorescence microscopy in the lower image. **C)** Regulation of self-incompatibility as a function of stigma maturation in developing floral buds. A developmental series of floral buds (top) and images of the self-pollinated stigmas from buds at similar stages of development (bottom) are shown. The stigmas are initially able to accept self-pollen shortly before flower opening. The light microscopy images in **A** and **B)** (top image) are reprinted from Nasrallah (2017), and the image in **B)** (bottom) is modified from Nasrallah (1997). The TEM images are reprinted from Kandasamy et al. (1994), and the floral-bud images are reprinted from Nasrallah and Nasrallah (2014). P, papilla; Po, pollen grain; PC, pollen coat; E, exine.

processes. While many pollen CRPs, like most other pollen coat proteins, are derived from the tapetum, some are synthesized in the male gametophyte or in both male gametophyte and tapetum (Schopfer and Nasrallah 2000; Wang et al. 2017).

Go signals at the pollen–stigma interface for compatible pollinations

Adhesion and hydration of the desiccated pollen grain

The pollen–stigma interaction is initiated by adhesion of the highly desiccated and metabolically inactive pollen grain to the stigma papilla. In *Arabidopsis thaliana*, the initial adhesion of pollen grains occurs within seconds of pollination and is highly selective, as heterospecific pollen grains adhere only weakly or not at all to papillae (Zinkl et al. 1999). Mutational and biochemical analyses have shown that this initial adhesion is mediated primarily by high-affinity chemical and/or biophysical interactions of exine polymers with the papilla surface and does not require an intact pollen coat (Edlund et al. 2004).

In response to contact with a compatible pollen grain, the papilla is rapidly induced to export water and other factors that are used by the pollen grain to fulfill the molecular

requirements of hydration, germination, and tube growth. As a critical checkpoint for pollination and fertilization, the process of pollen hydration has been the subject of intense study aimed at understanding the process of water release from the papilla and its uptake by the pollen grain, its complex mechanics, and especially the contribution of pollen and papilla factors to this process. The evidence obtained so far points to a complex molecular network of papilla-derived and pollen-derived factors that regulate pollen hydration.

Within minutes of its contact with a papilla, the pollen grain undergoes a dramatic change in its cellular water potential which increases its volume and transforms the oblong dehydrated grain into a spherical rehydrated grain (Fig. 1A). Transmission electron microcopy (TEM) images show that the viscous semi-fluid pollen coat material flows onto the surface of the papilla surface at the point of pollen contact (Fig. 1A; Kandasamy et al. 1994), resulting in an adhesion zone or attachment "foot" (Dickinson 1995) which strengthens pollen-papilla adhesion by anchoring the pollen grain to the papilla. This foot is the medium in which lipids and proteins of the pollen coat mix with molecules of the papilla surface. It also serves as the conduit for transfer of water along with nutrients and various other molecules from papilla to pollen grain and for exchange of signals between the grain and papilla. Indeed, isolated pollen coat can by itself induce in papillae the changes typically induced by contact with an intact pollen grain.

The essential role of pollen coat components in hydration has been clearly demonstrated by mutational analysis in *A. thaliana*. Mutant screens identified male-sterile plants whose pollen grains have an abnormal or very much reduced pollen coat and are severely impaired in their ability to hydrate and germinate. These phenotypes are caused by mutations in genes required throughout the plant for biosynthesis of very long-chain fatty acids (Edlund et al. 2004). The defective hydration phenotype of these mutants may be reversed by restoring hydraulic continuity between pollen and papilla either by supplying exogenous water in the form of increased humidity or by applying *cis*-unsaturated triacylglycerides to the stigma surface (Edlund et al. 2004). Because pollen tubes typically grow into the attachment foot and penetrate the papilla wall directly beneath the foot, it has been proposed that lipids transferred into the foot from the lipid-rich pollen coat are critical, not only for hydration of the pollen grain but also for setting up a water gradient that guides the pollen tube toward the hydrated papilla (Wolters-Arts et al. 1998).

Disruption of proteins of the pollen coat and in the pollen grain itself can also have a negative impact on pollen hydration. While loss of some proteins caused a delay in pollen hydration likely due to redundancy (Edlund et al. 2004), mutational depletion of the highly polymorphic Pollen Coat Protein-B (PCP-B) class of pollen coat CRPs abolishes the ability of pollen grains to hydrate, indicating that PCP-Bs function as major regulators of the pollen hydration checkpoint (Wang et al. 2017). In the pollen grain, aquaporins (water channels), which are known facilitators of bidirectional transport of water across membranes, have been implicated in the uptake of water by the pollen grain (Di Giorgio et al. 2016). Additionally, reactive oxygen species (ROS) regulate pollen hydration at least partly by controlling the activity of ion channels that sustain the pollen grain's ability to take up water from the stigma (Li et al. 2017). A pollen-specific cell wall pectin methylesterase is thought to promote pollen hydration by remodeling the intine pectin and increasing its hydrophilicity (Leroux et al. 2015). Additionally, a pollen-specific mechanosensitive ion channel senses the increased membrane tension produced by the rapid increase in water potential and responds, together with other regulators of turgor pressure, by adjusting osmolarity, thus maintaining cellular integrity in the rehydrating pollen grain (Hamilton et al. 2015).

In the papilla, several changes which are thought to promote water release for pollen hydration are rapidly induced by contact with a compatible pollen grain. Microscopic observations in *Brassica* and *Arabidopsis* have shown increased cellular activity in the region subtending the pollen attachment site, including an increase in cytosolic Ca^{2+}, accumulation of endoplasmic reticulum (ER), Golgi, and multivesicular bodies that fuse with the plasma membrane, and reorientation of the actin cytoskeleton in the direction of the pollen attachment site (Bosch and Wang 2020). The polarized secretion revealed by these studies has been associated with a targeted secretion process mediated by the exocyst, a complex that functions in exocytosis at sites of polarized secretion (Abhinandan et al. 2022). The nature of the cargo discharged by these vesicles is not known, but it presumably consists of largely undefined "compatibility" factors which are used for activities related to pollen hydration and for sustaining the metabolic requirement of pollen grains and their germinating tubes (Abhinandan et al. 2022). In parallel, a pathway for water transport out of the papilla uses an aquaporin localized in the ER which would regulate water transport out of the ER, and another aquaporin localized in the plasma membrane, which would regulate water transport to the cell's apoplast (Windari et al. 2021).

Undoubtedly, efficient water release requires modification of the papilla surface. Like other cells of aerial plant tissues, the papilla is protected against desiccation and other stresses by a lipophilic cuticle layer that restricts the diffusion of water, gases, and solutes. However, the papilla cuticle, unlike the cuticle of other plant cells, must allow rapid outflow of water in response to contact with a compatible pollen grain, and its chemical composition must be modified in the region subtending the site of pollen contact. The importance for pollen hydration of cuticle permeability is clearly illustrated by the ectopic hydration and germination of wild-type pollen grains on the leaf epidermal surfaces of *A. thaliana* transgenic plants that overexpress cutinase and of mutants whose cuticles have increased permeability due to aberrant composition (Edlund et al. 2004). Similarly, during flower development, a higher permeability of epidermal cell cuticles

in immature organs relative to mature organs might explain the ectopic hydration and germination of pollen grains on all epidermal surfaces in very young flower buds and subsequent growth of these tubes into underlying tissues (Kandasamy et al. 1994).

The pollen-induced release of water also requires active signaling in the papilla. Leucine-rich repeat receptor-like kinases expressed in stigmas have been identified which, when mutated in concert, result in reduced rates of pollen hydration (Abhinandan et al. 2022). A more dramatic effect was recently observed when ROS signaling was disrupted in the stigma (Liu et al. 2021). Stigmas accumulate ROS to substantial levels that peak when papillae are most receptive to pollen, and these ROS levels are reduced in stigmas pollinated with compatible pollen relative to unpollinated stigmas (McInnis et al. 2006). A molecular explanation for these observations and their connection to the role of PCP-Bs as regulators of pollen hydration was provided by experiments carried out in *A. thaliana* (Liu et al. 2021). Previous work had demonstrated that FERONIA (FER), a multifunctional receptor protein kinase, activates Rho-like GTPases (Guanosine Triphosphate Hydrolases) triggering RESPIRATORY BURST OXIDASE HOMOLOGS (RBOHs) to produce ROS (Cheung et al. 2022). Liu et al. (2021) demonstrated that a complex formed by 2 stigma-expressed receptor-like protein kinases, FER and ANJEA (ANJ), and the co-receptor Lorelei-like Glycosylphosphatidylinositol (GPI)-anchored protein 1 (LLG1) is activated by autocrine RAPID ALKALINIZATION FACTOR (RALF) peptides, leading to ROS production. Loss-of-function mutations in any one of the genes for the FER/ANJ to ROS pathway cause reduced ROS levels and more rapid pollen hydration relative to wild type stigmas. Because PCP-Bs also interact with the FER/ANJ receptor complex and displace RALF23/33 ligands from the complex at least in vitro, a model is proposed (Fig. 2; Huang et al. 2023) in which the PCP-Bs released by a compatible pollen grain successfully compete with RALFs for binding to the FER/ANJ complex which, with subsequent nitrosation of FER by compatible pollen-induced nitric oxide production, results in suppression of FER-mediated production of ROS, thus allowing pollen hydration and germination. It is not known if the reduction in ROS levels is localized to the region underlying the site of pollen contact. Also not known is how a decrease in ROS levels permits pollen hydration and if it affects the other cellular events that are triggered by compatible pollen.

Germination of the pollen grain and pollen tube growth in the papilla cell wall

In preparation for germination, the hydrated pollen grain is converted from a nonpolar to a highly polarized structure. Hydration is accompanied by an influx of Ca^{2+} mediated by a calmodulin-activated calcium pump which accumulates at the plasma membrane near the pollen attachment site (Bosch and Wang 2020). The establishment of polarity in the pollen grain involves several changes directed toward the future site of pollen tube emergence, including vesicle and actin polarization, and establishment of a cytoplasmic Ca^{2+} gradient which is required for elongation of the pollen tube by polar tip growth. These changes appear to be directed by a signal, possibly water or lipid as discussed earlier, emanating from the attachment foot, since the nascent pollen tube emerges precisely at the site of contact with the stigma papilla before growing within the attachment foot toward the papilla surface. Meanwhile, for pollen tube exit, the pollen grain's exine layer must be weakened by hydrolytic enzymes that may originate from the pollen grain or papilla.

Little is known about the molecular underpinnings of pollen tube entry into the papilla cell wall largely due to a dearth of relevant mutations that affect this process. Clearly, extensive enzymatic modification of the papilla surface is required. Indeed, even before arrival of the pollen tube at the papilla surface, signs of cuticle degradation and cell wall expansion are visible beneath the site of pollen contact (Dickinson 1995). However, little is known about the enzymes that cause these changes. At the point of pollen tube entry, the cuticle may be degraded by cutinases located in the stigma pellicle and by a pollen cutinase which may be activated by a factor located in the pellicle (Hiscock and Allen 2008). As for cell wall modifying enzymes, a possible pollen-expressed candidate is a pectin-degrading polygalacturonase whose levels increase at the pollen tube tip during its entry into the papilla cell wall (Dearnaley and Daggard 2001). And supporting these activities is a papilla expressed pollen coat-inducible auto-inhibited Ca^{2+} transporter which accumulates at the site where the pollen tube penetrates the papilla cell wall (Iwano et al. 2014). Papilla-specific factors are not apparently required for pollen tube entry into the cell wall once the cuticle layer is breached as indicated by the fact that pollen tubes can invade the epidermal cell walls of immature floral organs (Kandasamy et al. 1994).

Much research has focused on the mechanics of pollen tube growth and the cues and signaling networks that guide the tube through sub-epidermal tissues of the pistil (Cameron and Geitmann 2018; Johnson et al. 2019). Much less understood is how the pollen tube follows a straight path down the papilla cell wall toward the style. Some progress on this issue has been made by analysis of plants in which directional growth of the pollen tube in the papilla cell wall is lost. One study followed up on the observation that chemocyanin, a small extracellular protein belonging to the plantacyanin family of blue copper proteins, functions in directional guidance of the pollen tube in the transmitting tract of the lily pistil (Kim et al. 2003). Transgenic plants that overexpressed the sole plantacyanin gene in the *A. thaliana* genome did in fact have an aberrant pollination phenotype: when transformed stigmas were pollinated with wild-type pollen, pollen tubes grew toward the style, but only after making several turns within the papilla cell wall, and in some cases they even grew away from the style in the direction of the papilla cell apex (Dong et al. 2005). In a more recent study, a similar pollen tube coiling phenotype was

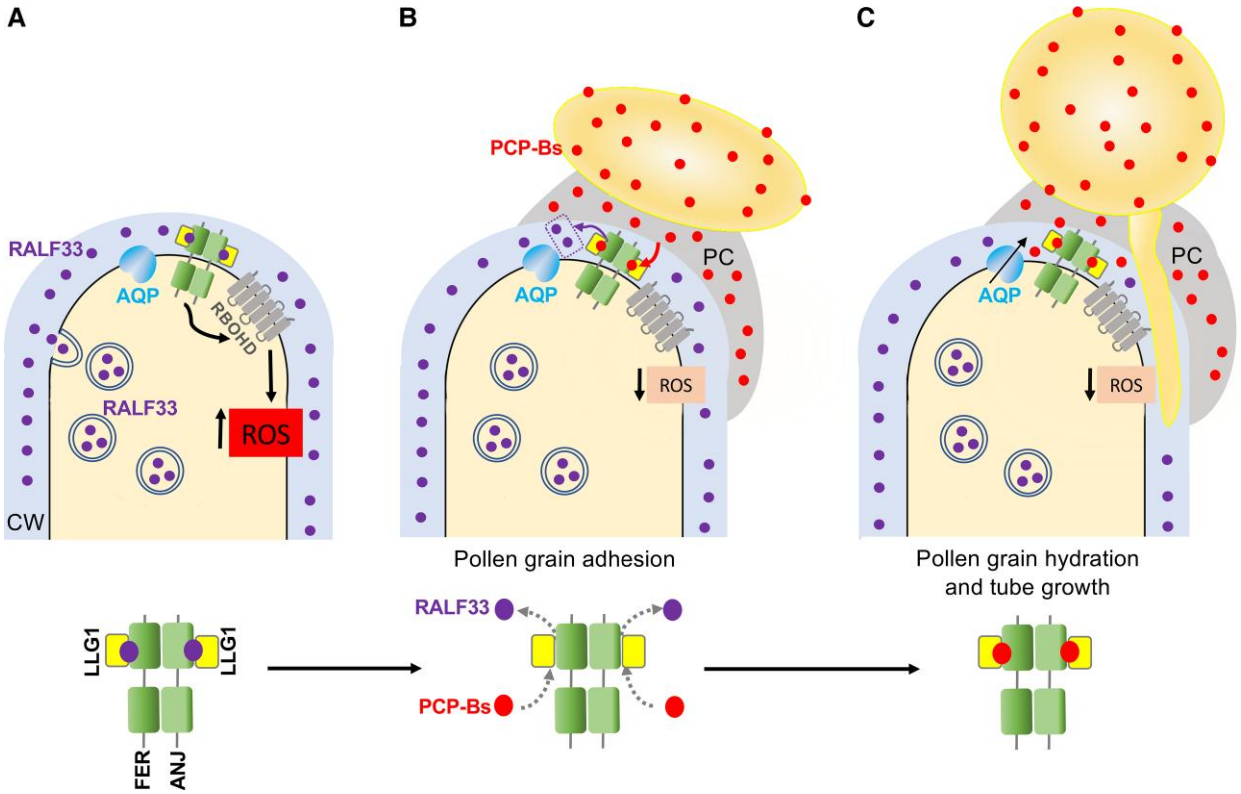

Figure 2. Proposed mechanism for hydration of compatible pollen grains based on the regulation of ROS levels by the FER/ANJ/LLG1 receptor complex. The figure shows a simplified diagram of this process in the papilla (upper diagrams) and a magnified view of the interaction of FER/ANJ/LLG1 with its ligands (lower diagrams). **A)** In the unpollinated stigma papilla of *A. thaliana*, the RALF33 peptide is secreted to the cell wall (CW) where it binds to the FER/ANJ/LLG1 complex and activates ROS production by RBOHD, thus maintaining high ROS levels. **B)** In a compatible pollination, pollen coat (PC) PCP-B peptides released from the pollen grain as the PC flows onto the papilla cell wall outcompete RALF33 for binding to the FER/ANJ/LLF1 complex causing reduced receptor signaling and low ROS levels. **C)** The reduced ROS levels caused by the compatible pollen–papilla interaction promote water transfer from papilla to pollen grain likely via aquaporin (AQP) channels, thus allowing pollen grain hydration, germination, and tube growth within the papilla cell wall. Not shown are intermediates of the FER/ANJ/LLG1-to-RBOH pathway, the reported increase in nitric oxide induced by compatible pollen with subsequent nitrosation of FER, and the various other cellular events known to be triggered by compatible pollination which are thought to promote water release from the papilla (see text). Note that while the displacement of RALF33 by PCP-Bs must occur in the region of the papilla that subtends the pollen grain as shown in **B** and **C),** it is not known how localized the effect of reduced FER/ANJ/LLG1 signaling is within the papilla.

observed in aging stigmas and in plants carrying mutations in a gene for katanin, a microtubule-severing enzyme which is the major regulator of cortical microtubule dynamics (Riglet et al. 2020). The pollen tube coiling phenotype was ascribed to a reduction in the overall stiffness of the papilla cell wall resulting from isotropic reorientation of cortical microtubules and associated cellulose microfibrils in the cell wall (Riglet et al. 2020). Therefore, both mechanical properties of the papilla cell wall and chemical guidance cues appear to contribute to directional growth of the pollen tube within the papilla cell wall.

Stop signals at the pollen–stigma interface for incompatible intraspecific and interspecific pollinations

For the crucifer stigma to fulfill its role as a selective sieve for pollen, 3 prezygotic pollen recognition systems must operate in tandem within the same papilla: (i) the compatible pollen recognition system described in the previous section, which provides a molecular environment conducive to pollen hydration, germination, and tube growth; (ii) in obligate outcrossers, a self-incompatibility system for discrimination between self- and nonself-conspecific pollen, which results in rejection of self-pollen, thus enforcing outcrossing and maintaining genetic diversity; and (iii) an interspecific incompatibility system for discrimination between conspecific and heterospecific pollen, which results in rejection of heterospecific pollen, thus allowing only legitimate unions, conserving pistil resources, and maintaining species integrity. Understanding the crosstalk between these recognition systems is the subject of intense study.

Contrasts and similarities between different self-incompatibility systems

The term "self-incompatibility" (SI) refers to a post-pollination pre-zygotic genetic barrier widespread among angiosperms

which ensures outcrossing by preventing selfing and mating among relatives. SI is typically controlled by loci that exist as multiple variants, each of which encodes a distinct mating (SI) specificity. The SI barrier is highly selective and is based on the ability of cells of the pistil to discriminate between self-pollen (i.e. from a pollen donor that expresses the same SI specificity as that expressed in the pistil) and nonself-pollen (i.e. from a pollen donor that expresses an SI specificity different from that expressed in the pistil). Pollen rejection occurs when SI specificities are matched in pistil and pollen, whether they are derived from the same flower, the same plant, or other plants that express the same SI specificity. Two major classes of SI systems have been described: heteromorphic SI systems which caught Darwin's attention (Darwin 1877) due to the association of different SI types with differences in floral morphology, such as the relative positions of stigmas and anthers (herkogamy), and homomorphic systems, in which different SI specificities cannot be distinguished based on floral morphology (Franklin-Tong 2008). The homomorphic SI systems of different families can differ with respect to the site and mode of pollen or pollen tube arrest, the number of genetic loci that control SI specificity, and the molecular basis of SI. These SI systems also differ in the genetic control of pollen SI specificity: sporophytic SI (SSI) in which specificity is controlled by the diploid genotype of the pollen-producing plant and gametophytic SI (GSI) in which specificity is controlled by the pollen grain's haploid genome.

Clearly, SI evolved independently in different lineages. The use of the term "S locus" (for "Sterility locus") to designate unrelated SI specificity-determining genetic loci is an unfortunate relict of early genetic studies that predated the molecular identification of SI recognition genes. The molecular data also discredit the notion espoused by early researchers that the SSI and GSI categories each encompasses related SI systems that use the same mechanism for recognition and rejection of incompatible pollen. Rather, molecular analysis of SI in the families investigated to date underscores a key distinction between GSI and SSI systems: namely, which structure, pistil or pollen, produces the signal that triggers the SI rejection pathway (the pistil in GSI and pollen in SSI) and which cell receives and transduces this signal into a cellular response that culminates in pollen arrest (pollen in GSI and pistil in SSI).

Despite the variability of SI mechanisms, several features are shared by different homomorphic SI systems. The number of SI variants is typically large as expected for systems in which allelic diversity is maintained by negative frequency-dependent selection where new and therefore rare alleles have a reproductive advantage and will increase in frequency in a population. And as expected for variants that persist in species for long periods of time due to strong heterozygote advantage, these recognition genes typically exhibit an extraordinarily high level of intra-specific sequence polymorphisms and frequent trans-specific polymorphisms. In all cases analyzed to date, the genetic loci that control SI specificity consist of separate coadapted recognition genes that encode the pistil and pollen determinants of SI specificity, the former expressed at the site in the pistil of pollen or pollen tube arrest, and the latter expressed in tapetum (SSI) or pollen tube (GSI). And it is the allelic combination of pistil and pollen recognition genes which forms a nonrecombining genomic segment or haplotype (Nasrallah and Nasrallah 1993) which determines pistil and pollen SI specificity.

General features of SI in crucifers

In crucifers, the rejection of self-pollen is primarily due to the failure of pollen hydration although a few self-pollen grains can hydrate and germinate (Fig. 1B). The genetic control of crucifer SI by a single multi-allelic "S" locus with sporophytic control of pollen SI phenotype was demonstrated in the 1950s by classical genetic studies in *Brassica* and *Raphanus* among other species (Bateman 1955; Thompson 1957). Further studies showed the existence in any one species of many distinct SI specificities or S haplotypes, numbering up to 100 in some species (Lawrence 2000). Moreover, diallel crosses revealed that complex genetic interactions between S haplotypes in S-locus heterozygotes underlie the specification of SI phenotype, not only in stigmas but also in pollen, as expected from sporophytic control of pollen SI phenotype. In parallel, the SI response was found to be regulated over the course of stigma maturation, whereby stigmas are initially unable to discriminate between self- and nonself-pollen, and only acquire the ability to reject self-pollen at the time anthers release their pollen and before the flower opens (Fig. 1C; Nasrallah 1974). This developmental regulation has been invaluable for mechanistic studies of SI and for SI-based breeding programs because it allows the production and maintenance of S-locus homozygotes by bud pollination, i.e. manual pollination of self-compatible stigmas before the onset of SI.

Molecular basis of self-pollen recognition: selective activation of stigma receptors by pollen-borne ligands

Early attempts to identify the determinants of SI specificity in crucifers, like attempts in other species (Lewis 1952), initially focused on the pollen SI determinant and were unsuccessful. It was only when the focus shifted from pollen to stigma that progress was achieved by M.E. Nasrallah in the D.H. Wallace laboratory at Cornell University (Nasrallah and Wallace 1967). The expectation was that a candidate stigma SI determinant would exhibit several characteristics: a high degree of intraspecific polymorphism, genetic linkage to the S locus, expression in stigma papillae, and a developmental regulation pattern that parallels the developmental regulation of SI over the course of stigma maturation. A protein having these expected features was identified in stigma extracts using immunochemical methods (Nasrallah and Wallace 1967). This protein, now designated S-locus glycoprotein

(SLG), is an abundant secreted glycoprotein that localizes to the papilla cell wall (Kandasamy et al. 1989) and is unrelated to other proteins known at the time of its discovery (Nasrallah et al. 1987). While SLG was later found not to be the sought-after stigma SI determinant, it was key for the eventual identification of the actual stigma determinant. It turned out that SLG belongs to a gene family, one member of which proved to be an S-locus linked highly polymorphic gene expressed in stigmas (Stein et al. 1991). This gene, called the *S-locus Receptor Kinase* (*SRK*) gene would become the prototypic member of the so-called S-domain receptor like kinase (SD-RLK) family which includes RLKs and receptor-like proteins (SD-RLPs) like SLG (Fig. 3, A and B). Importantly, SRK itself was the starting point for a chromosome walking strategy which allowed the identification of an anther-expressed gene that encodes the small and extremely polymorphic S-locus cystine-rich (SCR) protein (Schopfer et al. 1999; Schopfer and Nasrallah 2000). Mutant analysis and transgenic experiments have demonstrated that SRK and SCR are respectively the stigma and pollen determinants of SI specificity (Schopfer et al. 1999; Takasaki et al. 2000). Thus, SRK bestows on the stigma the ability to distinguish self- from nonself-pollen grains, and SCR identifies the pollen grain as being self or nonself, thus marking it for rejection or acceptance.

The *SRK* gene is expressed predominantly in papillae and to a lesser extent in the transmitting tissue of the style, and its expression attains peak levels coincident with the onset of SI in mature bud stigmas (Stein et al. 1991). The SRK protein consists of an N-terminal extracellular glycosylated S-domain (designated eSRK), a single-pass transmembrane domain, and a C-terminal kinase domain (Fig. 3B). Consistent with its overall structure, SRK is an integral protein of the plasma membrane of the papilla (Fig. 3B; Stein et al. 1996; Rea and Nasrallah 2015). Indeed, this localization is critical for the stigma's ability to recognize and arrest self-pollen, as this ability is abrogated when SRK is trapped within the ER (Yamamoto et al. 2014; Tantikanjana and Nasrallah 2015). Within an *S* haplotype, the *eSRK* and *SLG* share a high degree of sequence similarity, suggesting that *SLG* was generated by partial duplication of *SRK* (Stein et al. 1991). This similarity might explain why SLG can enhance the stigma SI response triggered by its cognate SRK (Takasaki et al. 2000), possibly by contributing in a haplotype-specific manner to the proper maturation and subcellular targeting of SRK (Dixit et al. 2000).

For its part, the small (typically ~50 amino acids in length) SCR protein (also known as SP11), is synthesized in the anther tapetum and subsequently transferred to the outer coating of the pollen grain (Schopfer and Nasarallah 2000; Kachroo et al. 2001; Iwano et al. 2003). Despite their extreme sequence divergence, SCR variants assume the same overall 3D structure consisting of a defensin-like cystine-stabilized αβ-fold (Fig. 3C). Biochemical analysis has demonstrated that SCR binds the eSRK (Kachroo et al. 2001; Takayama et al. 2001) and high-resolution 3D structural analysis has shown that SCR induces the formation of a heterotetramer consisting of 2 eSRK and 2 SCR molecules (Ma et al. 2016). Both types of analyses also demonstrated that the eSRK-SCR interaction is *S* haplotype-specific and will occur only when receptor and ligand are encoded in the same *S* haplotype. As a component of the pollen coat, SCR is carried to the papilla surface via the attachment foot and transferred across the papilla cuticle and cell wall toward the plasma membrane where it gains access to the eSRK (Iwano et al. 2003). While these processes occur in both self- and cross-pollination, it is only in a self-pollination that the SCR ligand will bind and activate the SRK receptor. This haplotype-specific interaction forms the basis of selective recognition of self-pollen, resulting in autophosphorylation of SRK and the triggering by the activated receptor of a signaling cascade within the papilla which culminates in arrest of self-pollen. Notably, the self-pollen grain is not killed in the process (Kroh 1966), which is different from the GSI response of the Solanaceae and Papaveraceae, in which self-pollen tubes are targeted for destruction by a signaling pathway triggered within the pollen tube (Franklin-Tong 2008).

Sporophytic control: solving the puzzle of dominance-recessiveness

A hallmark of the crucifer SSI system is the complex genetic interactions between *S* haplotypes (designated S_1, S_2, S_3 … S_n) which determine SI phenotype in both stigma and pollen. Interactions of codominance, dominance/recessiveness, incomplete dominance, or mutual weakening occur. And these interactions can differ in the specification of SI phenotype in stigma and pollen consistent with distinct molecules determining specificity in the two interacting partners. While most allelic interactions are codominant, dominant-recessive interactions forming complex nonlinear dominance series are frequently observed in the determination of pollen SI phenotype (Thompson 1957). Notably, dominant-recessive relationships can impact the frequencies attained by *S* haplotypes in a population. Thus, "pollen recessive" haplotypes attain high frequencies because they allow pollen to evade the stigma-mediated rejection of self-pollen and they can therefore occur in homozygous condition in nature.

The molecular basis of these genetic interactions is best understood for "pollen recessive" *S* haplotypes. Because of sporophytic control, in plants heterozygous for 2 codominant *S* haplotypes (e.g. S_1 and S_2), the haploid pollen grains are genotypically S_1 or S_2 but phenotypically S_1S_2 because their pollen coat contains both SCR_1 and SCR_2. However, if the S_1 haplotype is dominant to the S_2 haplotype, all pollen grains will be phenotypically S_1 whether they are genotypically S_1 or S_2. As shown in *A. lyrata* (Kusaba et al. 2002) and *Brassica rapa* (Shiba et al. 2002), these dominant-recessive interactions result from monoallelic expression of the dominant *SCR* allele due to epigenetic silencing of the recessive *SCR* allele. This silencing is achieved by *S*-locus-linked small-RNAs found in dominant haplotypes which target homologous sequences near *SCR*

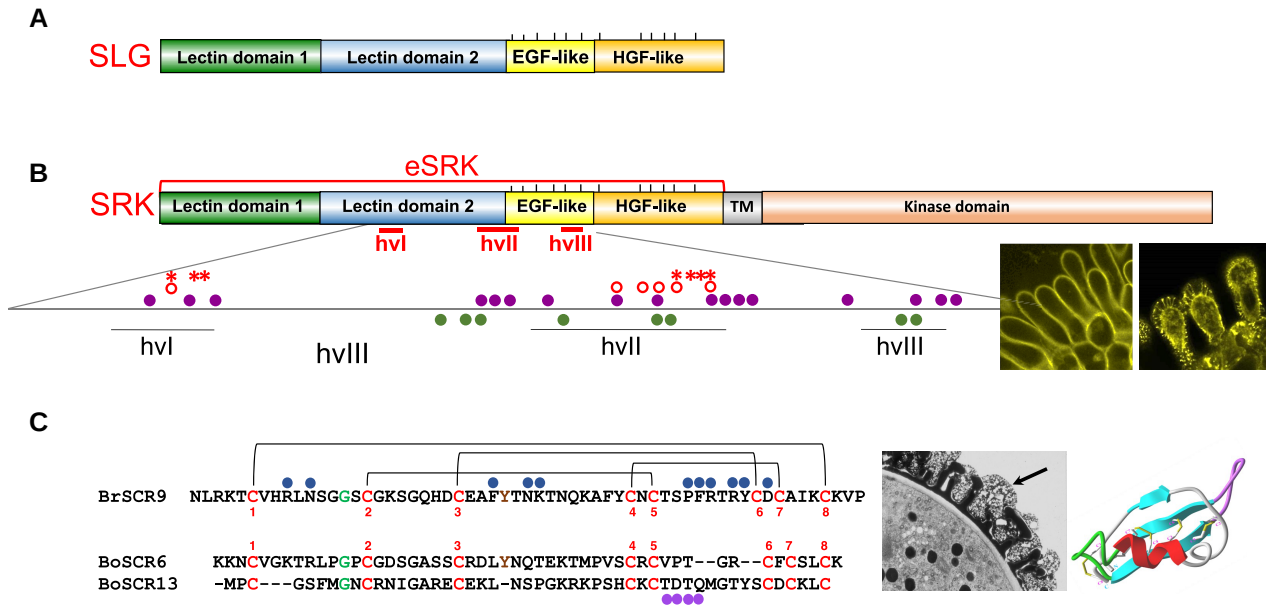

Figure 3. Structure of *S* locus-encoded proteins. **A)** Structure of the SLG protein. The diagram shows the modular organization that characterizes the S-domain in members of the SD-RLK/RLP family. The S-domain consists of 2 N-terminal lectin domains, an epidermal growth factor (EGF)-like domain, and a hepatocyte growth factor (HGF)-like domain. The vertical lines above the diagram mark the twelve cysteine residues that are conserved in S-domain proteins. The *SLG* gene occurs in most *S* haplotypes from *Brassica* and *Raphanus* spp. but not in *S* haplotypes from *Arabidopsis* and *Capsella* spp. **B)** Structure of the SRK protein. The protein consists of an extracellular S domain (eSRK), a transmembrane domain (TM), and a kinase domain. The location of the 3 hypervariable (hv) regions that contain the SI specificity determinants and the amino acids that form the SRK-SCR binding pocket are shown. Below the diagram is an enlarged view of the hv-containing segment that shows the location of amino-acid residues found to be critical for SRK function. The amino-acid residues essential for *in-planta* activation of SI in 2 SRK variants are shown by open circles for one variant and asterisks for the second variant (Boggs et al. 2009c). The enlargement also shows the amino acids that form contact points with SCR9 (filled circles above the line) and those involved in eSRK9 homodimerization (filled circles below the line) as determined by the 3D crystal structure of the $eSRK_9$-SCR_9 tetrameric complex. Note that residues in hvIII were not tested for activity *in planta*. To the right of the diagram are confocal images of an $AlSRK_{20}$-YFP fusion protein which confers SI in *A. thaliana* C24 stigmas. The images show that the $AlSRK_{20}$-YFP signal is primarily localized at the periphery of papillae (left) and in the plasma membrane and hechtian strands in plasmolyzed papillae (right). **C)** Structure and extreme diversity of the SCR protein. Sequence alignment of *B. rapa* SCR_9 (BrSCR9) and *B. oleracea* SCR_6 (BoSCR6) and SCR_{13} (BoSCR13). The conserved cysteines are shown joined by 4 di-sulfide bridges (brackets above the BrSCR9 sequence). The sequences show the conserved glycine residue and the aromatic residue between C3 and C4 which is conserved in most SCRs including BrSCR9 and BoSCR6 but is missing in BoSCR13. BrSCR9 amino acids that form contact points with eSRK9 in the crystal structure of the *B. rapa* eSRK9-SCR9 complex are indicated by filled circles above the BsSCR9 sequence. The TDTQ amino acid sequence in BoSCR13 which determines S_{13} specificity is indicated by filled circles below the BoSCR6 and BoSCR13 sequence alignment. Note that the specificity determinants in SCR6 could not be identified either because they are not located exclusively in the same region as SCR13 determinants or because residues outside this region affect the conformation of the SRK binding pocket. To the right of the alignments is a TEM image of the pollen grain with the arrow pointing to the pollen coat and a 3D model of the BoSCR13 protein showing a structure composed of an α-helix and a triple-stranded antiparallel β-sheet forming a defensin-like cystine-stabilized αβ structure. The SCR_{13} specificity-determining TDTQ sequence is located in the unstructured loop that projects from the core of the 3D model. The confocal images in **B)** are reprinted from Tantikanjana and Nasrallah (2015). The 3D model of BoSCR13 in **C)** is reprinted from Chookajorn et al. (2004).

genes in the recessive haplotypes, resulting in methylation and inactivation of the recessive *SCR* gene (Tarutani et al. 2010). This homology-based small-RNA system for control of SCR dominance relationships appears to be conserved across crucifers (Fujii and Takayama 2018). It was shown to operate in *Brassica* (Tarutani et al. 2010) and *Arabidopsis* (Durand et al. 2014; Yasuda et al. 2021) spp., although the number of sRNAs involved can vary according to the complexity of the dominance hierarchy in various species. In one case, analysis of the linear dominance series of six *A. halleri* *S* haplotypes identified at least 17 *S*-locus-linked small-RNA genes and their respective targets which together control the dominance hierarchy of these haplotypes (Durand et al. 2014).

By contrast, the basis of dominant-recessive *S* haplotype interactions in the stigma is not understood, as no silencing or drastic dampening of expression of the recessive *SRK* allele has been observed in the stigmas of *S*-locus heterozygotes. Evidence suggests that SRK forms homodimers in the absence of its ligand *in planta* (Giranton et al. 2000) and that this dimerization is critical for high-affinity binding to its cognate SCR (Shimosato et al. 2007). It has been suggested that the 2 SRKs expressed in heterozygous stigmas may differ in their propensity to form ligand-independent homodimers

and heterodimers, and that these heteromers may differ in their affinity for their SCR ligand as reported for other receptor-ligand systems (Naithani et al. 2007). In this scenario, codominance in an S_1S_2 stigma would result from a strong preference of both SRK_1 and SRK_2 for forming homodimers having strong affinity for their respective SCR ligands, while a dominant-recessive interaction in an S_3S_4 stigma might result from an increased propensity of the recessive SRK, e.g. SRK_4, for forming SRK_3-SRK_4 heterodimers having weak affinity for the SCR_4 ligand but retaining strong affinity for the SCR_3 ligand (Naithani et al. 2007).

Genetic linkage and coevolution at the S locus: keeping partners together

Given that different SI specificities are defined by combinations of matched allelic forms of *SRK* and *SCR*, the proper functioning and long-term preservation of SI depends on the coevolution of SRK/SCR proteins to ensure their structural compatibility and on maintenance of the *SRK-SCR* gene complex in a tightly linked genetic unit. Several features of SRK and SCR indicate that the 2 proteins coevolve: their S haplotype-specific interaction, the fact that the crystal structures of the eSRK in complex with self-SCR show strong interaction interfaces while models of eSRK-nonself-SCR complexes reveal steric clashes (Ma et al. 2016; Murase et al. 2020), and the congruence of their gene trees (Sato et al. 2002; Goubet et al. 2012). Regarding genetic linkage, the expectation is that recombination events that disrupt the linkage of coadapted *SRK-SCR* gene pairs will produce nonfunctional haplotypes, resulting in the breakdown of SI. Indeed, *SRK* and *SCR* are in strong linkage disequilibrium, which is likely due to the extensive intraspecific differences in overall organization and sequence content exhibited by S haplotypes (Fig. 4, A and B; Boyes et al. 1997; Kusaba et al. 2001; Goubet et al. 2012). The haplotypes can vary significantly in overall physical size, in the orientation of their *SRK* and *SCR* genes relative to each other, and in the physical distance between the 2 genes and relative to flanking genes. They also contain haplotype-specific sequences, and they differ in the number and families of transposable elements they harbor. Indeed, the S haplotype is easily recognizable within the genome as a highly variable and rearranged segment flanked by conserved colinear regions (Fig. 4A). Since S haplotypes typically exist in heterozygous condition, these features would prevent proper DNA pairing during meiosis, thus causing suppression of recombination in the S haplotype and maintaining the linkage of coadapted allelic combinations of *SRK* and *SCR* genes over time. Similar rearranged gene order, fractured homology, and suppressed recombination are common features of genomic regions containing coadapted gene complexes, such as the mating type loci of fungi and green algae (Ferris and Goodenough 1994; Hartmann et al. 2020), sex-determining chromosomes (Charlesworth 2023), histocompatibility loci in mammals and invertebrates (Cadavid et al. 2004; Nasrallah 2005), and loci for disease resistance in plants (Schweiger et al. 2016).

Establishment of a transgenic self-incompatible *A. thaliana* model for mechanistic and evolutionary studies of SI

To circumvent the difficulties associated with analysis of SI in nonmodel *Brassica* spp., attempts were made to transfer the SI trait to the highly self-fertile *A. thaliana* which has well-known advantages for molecular genetic studies. Initial attempts using transformation with Brassica *SRK-SCR* genes were unsuccessful, which was taken to mean that a complex trait like SI could not be transferred to a self-fertile species by direct transformation with only 2 genes (ME Nasrallah, unpublished data). Consequently, another approach was used which involved crossing *A. thaliana* and *A. lyrata* despite the known problems associated with inter-specific crosses, especially those involving species having different chromosome numbers such as the 2 *Arabidopsis* spp. being crossed (Nasrallah et al. 2000). It was only after *S* haplotypes from self-incompatible *A. lyrata* were analyzed and their *SRK* and *SCR* genes were identified (Kusaba et al. 2001) that transfer of the SI trait to *A. thaliana* was realized.

In the first transformation experiment, the *AlSRK* and *AlSCR* genes isolated from the *Sb* (hereafter S_{20}) haplotype of *A. lyrata* were introduced into the Col-0 accession, resulting in $AlSRK_{20}$-$AlSCR_{20}$ transformants that expressed a transient SI phenotype: an SI response was evident in young floral buds during a narrow window of stigma development but broke down in older flowers due to suboptimal levels of $AlSRK_{20}$ transcripts (Nasrallah et al. 2002). Despite setting abundant seed, the Col-0[$AlSRK_{20}$-$AlSCR_{20}$] plants exhibit as strong an inhibition of self-pollen in their younger stigmas as that observed in *A. lyrata* S_{20} stigmas. Subsequent transformations of other *A. thaliana* accessions produced different outcomes (Fig. 4C; Boggs et al. 2009a; Dwyer et al. 2013). Some accessions exhibited weak SI while others, such as the Lz accession, failed to express SI at any stage of stigma development despite exhibiting high and developmentally stable expression of the *SRK* gene (Fig. 4C), suggesting that they may carry mutations in the SRK signaling pathway. Importantly, *SRK-SCR* transformants of 5 accessions (C24, Cvi-0, Hodja, Kas-2, Sha) do express a developmentally stable SI response that persists in older flowers and consequently set very few seeds (Fig. 4C; Nasrallah et al. 2004; Boggs et al. 2009a; Nasrallah and Nasrallah 2014). This reversal of *A. thaliana* from autogamy to full SI indicates that the species has retained a functional SRK-mediated pollen rejection pathway. It also provides further evidence that SRK and SCR are the sole determinants of SI in crucifers and demonstrates that these 2 proteins are the primary determinants of the outcrossing mode of mating in crucifers.

Transgenic self-incompatible *A. thaliana* plants have now been successfully generated using different *SRK-SCR* gene pairs isolated from *A. lyrata*, *A. halleri*, and *Capsella*

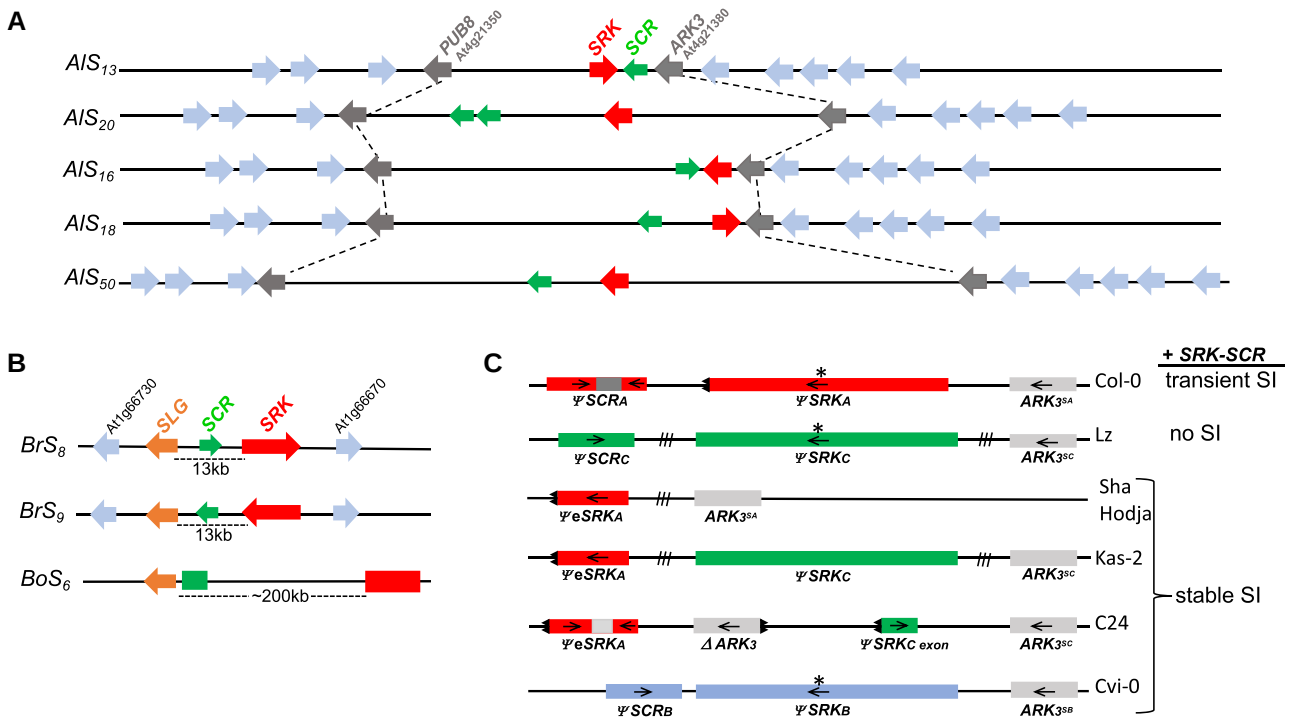

Figure 4. Structural heteromorphism of *S* haplotypes. The figure shows the rearranged gene order of representative *S* haplotypes from *Arabidopsis* and *Brassica* spp. **A)** *S* haplotypes from self-incompatible *A. lyrata*; **B)** *S* haplotypes from self-incompatible *B. rapa* (*Br*) and *B. oleracea* (*Bo*); **C)** non-functional *S* haplotypes from several *A. thaliana* accessions. The arrows indicate the 5′→3′ orientation of each gene, while boxes with no arrows indicate that gene orientation is not known. The diagrams are not drawn to scale. **A** and **B)** The *Brassica S* haplotypes contain an *SLG* gene while *A. lyrata* haplotypes do not. In *Arabidopsis* spp. and *Capsella* spp. (not shown), the *S* haplotype is located in a region that corresponds to the segment flanked by the *PUB8* (At4g21350) and *ARK3* (At4g21380) genes on chromosome 4 of *A. thaliana*. The *Brassica S* locus was translocated to a genomic location that corresponds to a region on chromosome 1 of *A. thaliana* (Conner et al. 1998). The *S* haplotypes differ in their overall size and with respect to the distance between *SRK* and *SCR* and the orientation of these genes relative to each other and to flanking genes. This rearranged gene order can be contrasted with the collinearity of genes that flank the *S* haplotype in *Arabidopsis* spp. **C)** The organization of the nonfunctional *S* haplotypes in several *A. thaliana* accessions are shown along with the pollination phenotype conferred by the $AlSRK_{20}$-$AlSCR_{20}$ transgenes in each accession. Sequences from the 3 *S* haplogroups, S_A, S_B, and S_C, which have been retained in the species are shown. The *S* haplotypes carry inactivating substitutions (asterisks), deletions, inversions, or inter-haplogroup recombination events. $AlSRK_{20}$-$AlSCR_{20}$ transformants of the C24, Sha, Hodja, Kas-2, and Cvi-0 accessions exhibit complete reversion to SI, indicating that inactivation of the *S* locus caused loss of SI in these accessions. Col-0[$AlSRK_{20}$-$AlSCR_{20}$] transformants exhibit transient SI due to suboptimal levels of SRK at later stages of sigma development, while Lz[$AlSRK_{20}$-$AlSCR_{20}$] plants do not express SI at any stage of stigma development despite expressing adequate levels of $AlSRK_{20}$ and $AlSCR_{20}$ transcripts, possibly due to the presence of mutation(s) in the SI pollen rejection pathway. Arrows show the 5′→3′ orientation of the sequences and teeth marks indicate 5′ and 3′ gene truncations. Hatch marks between the genes or gene fragments indicate the segments for which length and sequence content are not known. The maps were drawn according to Kusaba et al. (2001) for AlS_{13} and AlS_{20} (previously referred to as *AlSa* and *AlSb*, respectively), Guo et al. (2011) for AlS_{16} and AlS_{50}, and Goubet et al. (2012) for AlS_{18}. **C)** was modified from Nasrallah (2017).

grandiflora (Nasrallah et al. 2002, 2004; Boggs et al. 2009b; Dwyer et al. 2013; Durand et al. 2014; Chantreau et al. 2019; Zhang et al. 2019; Rozier et al. 2020). It should be noted that not all *SRK-SCR* gene pairs can confer SI in *A. thaliana*. All *Brassica SRK-SCR* gene variants tested to date (Zhang et al. 2019; Yamamoto et al. 2022; ME Nasrallah, unpublished data) and even some *SRK-SCR* gene pairs from *A. lyrata*, a close relative of *A. thaliana*, failed to restore SI due to the inability of the *SRK* gene to function in *A. thaliana* (Boggs et al. 2009b). The range of species from which these ineffective SRKs are derived indicates that there is no genus-specific preference for *SRK-SCR* gene pairs, contrary to the claim of Zhang et al. (2019). Indeed, the functionality of some, but not all, SRKs from *A. lyrata*, *C. grandiflora*, and *B. rapa* could be restored (Boggs et al. 2009b; Yamamoto et al. 2022) using a strategy developed by Nathan Boggs in which SRK chimeras (SRKx-SRK_{20}^{kin}) are generated by replacing the native kinase domain with the SRK_{20} kinase domain (Boggs et al. 2009b). The reason for the inactivity of some SRKx-SRK_{20}^{kin} chimeras despite a kinase domain of proven effectiveness may vary among chimeras; one possibility is that they may not be properly targeted to the plasma membrane as reported for 2 *B. rapa* chimeras (Yamamoto et al. 2022). In any case, as described in the next sections, the transgenic self-incompatible

A. thaliana platform has proven to be invaluable for mechanistic studies of the recognition and response phases of the SI response and for addressing evolutionary questions related to SI and its loss in transitions to self-fertility.

S-locus polymorphisms and genesis of the SI recognition repertoire

SI recognition genes, like those of other self/nonself-discrimination systems, have been shaped by selective pressures for diversification and coevolution of recognition functions. In crucifers, the SI recognition repertoire has been fashioned over millions of years of evolution. Consistent with the very old age of *S* haplotypes, which predate speciation in the family, trans-specific and even trans-generic polymorphisms in SI specificity and *SRK/SCR* gene sequences are common (Dwyer et al. 1991; Sato et al. 2002; Guo et al. 2011; Goubet et al. 2012). Understanding the diversification of *S* haplotypes and the molecular basis of the haplotype-specific eSRK-SCR interaction requires identification of the specificity-determining amino-acid residues in receptor and ligand. However, identification of these residues is complicated by the extremely high levels of intraspecific sequence divergence of these proteins. The eSRKs can exhibit as much as 35% amino acid divergence in *Brassica* spp. and ~40% to 50% in *A. lyrata*, and many of the polymorphic residues show signals of positive selection as expected for determinants of recognition specificity (Sainudiin et al. 2005). With a few exceptions, SCR alleles are even more diverged: only the core 8 cysteines, a glycine residue, and an aromatic residue are conserved in most variants, while the regions between the cysteines often differ in length and typically exhibit little if any sequence similarity (Fig. 3C).

The polymorphic residues in the eSRK are scattered over the length of the protein but are particularly concentrated in several "hypervariable regions" (Fig. 3B; Boggs et al. 2009c). However, determining which suspected regions or residues in eSRK and SCR are *bona fide* determinants of SI specificity requires in vivo structure-function analysis of eSRK and SCR variants engineered by inter-allelic domain and single-residue swaps. For SCR, assays in which engineered SCRs were applied to stigmas expressing the relevant SI specificity showed that specificity determinants can consist of a cluster of as few as 4 residues and surprisingly, they can occur in different regions of the protein or can consist of combinations of regions in different SCRs (Fig. 3C; Chookajorn et al. 2004).

In the case of SRK, identification of SI specificity residues requires *in planta* analysis of engineered eSRKs reconstituted into full-length SRK receptors. Once several SI specificities were expressed in *A. thaliana* C24 plants (Boggs et al. 2009b), it was possible to generate the required SRK variants for expression in *A. thaliana*. Unexpectedly, even though approximately half of the amino acids in the eSRK exhibit significant levels of polymorphism among *S* haplotypes, the specificity determinants were found to consist of only a small number of amino-acid residues located in 2 noncontiguous clusters in the hvI and hvII regions (Fig. 3B; Boggs et al. 2009c). The inference that these 2 clusters are brought into close juxtaposition in the 3D structure of the eSRK to form an SCR-binding pocket was validated by the crystal structure of the *B. rapa* $eSRK_9$-SCR_9 complex (Ma et al. 2016). In fact, there is significant overlap between the location of the specificity-determining residues identified *in planta* for *A. lyrata*- and *C. grandiflora*-derived sequences and the position of amino-acid residues that form contact points between the eSRK and SCR in the 3D structure of the *B. rapa* $eSRK_9$-SCR_9 complex (Fig. 3, B and C). Although it is likely that roughly the same amino-acid regions form the eSRK-SCR interaction interface between various eSRK-SCR pairs, the divergence of these proteins is such that the exact mode of the interaction can differ among different SRK/SCR variants (Murase et al. 2020). Nevertheless, the structure-function studies of eSRK and SCR indicate that most polymorphic residues are not essential for SI specificity as expected. Indeed, because of the very old age of *S* haplotypes and suppressed recombination at the *S* locus, amino-acid substitutions are expected to accumulate in SRK and SCR at sites near their specificity determinants. Additionally, specificity determinants retain functionality in the context of highly diverged backbones and acquisition of a novel specificity can result from a small number of amino-acid changes in the 2 proteins (Chookajorn et al. 2004; Boggs et al 2009c; Chantreau et al. 2019).

An important question is how new SI specificities are generated in a 2-component SI system (Charlesworth 2000). For crucifers, the conundrum is that for a new SI specificity to arise, the *SRK* and *SCR* genes contained in the same *S* haplotype must be modified coordinately and not only by a single mutation but by multiple mutations, as indicated by *in planta* structure function and 3D structure analyses (Fig. 3, B and C). Several hypotheses have been proposed to explain how this process has occurred repeatedly in nature to generate large numbers of SI specificities. Among these, is a hypothesis that proposes passage through self-compatible (SC) intermediates. According to this hypothesis, a change in one gene which disrupts the SRK-SCR interaction and produces a nonfunctional *S* haplotype is followed by a compensatory change in the second gene within the same haplotype which would restore the interaction as long as it occurs before the nonfunctional *S* haplotype carrying the first change is lost from the population (Uyenoyama et al. 2001). An alternative hypothesis proposes that new SI specificities are generated through self-incompatible intermediates by small gradual readjustments of the SRK-SCR interaction without disrupting the interaction or signaling outcome, resulting eventually in the splitting of a new specificity while retaining the old one (Chookajorn et al. 2004). Efforts are being made to obtain evidence for or against these models (Chantreau et al. 2019; Durand et al. 2020).

The response phase of SI: multiple pathways for pollen rejection?

Contact with self-pollen triggers rapid cytological changes in the stigma papilla. These changes include callose deposition

beneath the site of pollen contact accompanied by rapid increase in cytosolic Ca^{2+}, actin depolymerization, disorganization of the vacuole, and inhibition of vesicular trafficking (Bosch and Wang 2020). Together, these changes are thought to disrupt secretion of compatibility factors and water release from the stigma. Because most self-pollen grains fail to hydrate, studies of SRK-mediated signaling have rightly focused on the pollen hydration checkpoint. As a result, another feature of self-pollen rejection has been largely ignored, namely that when self-pollen grains breach the hydration checkpoint and germinate, their pollen tubes will either fail to enter the papilla cell wall (Fig. 1B) or they will be arrested shortly after entering the wall. It is not known what factors regulate these additional checkpoints.

The nature of the proteins that have been proposed to function in SRK-mediated signaling is consistent with the expectation that this pathway would override the compatible pollen acceptance pathway. In *B. napus*, a yeast 2-hybrid screen for proteins that interact with the kinase domain of SRK identified the ARM-repeat and U-box protein ARC1 which itself interacts with the Exo70A1 subunit of the exocyst (Abhinandan et al. 2022). Based on these findings, a signaling pathway was proposed in which SRK, activated by its interaction with self-SCR, phosphorylates ARC1, which then ubiquitinates and targets Exo70A1 for degradation by virtue of its E3 ligase activity. As a result, vesicular trafficking and the polarized secretion would be disrupted and compatibility factors would be targeted for degradation instead of being secreted by the papilla (Abhinandan et al. 2022). In a separate study, analysis of a self-fertile *B. rapa* cultivar identified the membrane-localized protein kinase MLPK as a potential effector of SRK (Kakita et al. 2007). However, MLPK function is not required for the operation of at least one *S* haplotype in *B. rapa* (Ohata et al. 2023). Moreover, neither the SRK-ARC1-Exo70A1 pathway nor MLPK are required for the SI response in transgenic self-incompatible *A. thaliana* plants (Kitashiba et al. 2011; Nasrallah and Nasrallah 2014). Indeed, the *ARC1* gene is deleted or only present as a nonfunctional fragment in the genomes of *A. thaliana* accessions, and neither loss-of-function mutations in the closest paralog of *MLPK* nor overexpression of Exo70A1 in *A. thaliana SRK-SCR* transformants caused the weakening of the SI response reported in most analyzed *Brassica* strains (Kitashiba et al. 2011; Table 2).

Another as-yet unexplained discrepancy between *Brassica* and *Arabidopsis* spp. relates to the induction of autophagy in self-pollinated papillae (Abhinandan et al. 2022): autophagosomes, which are proposed to function as an additional process for clearing compatibility factors, have been observed in the papillae of self-incompatible *Arabidopsis* plants but not in those of *Brassica* (Table 2). Setting aside the curious intraspecific difference regarding the requirement of MLPK in SI (Ohata et al. 2023), two possible explanations for the discrepancies observed between *Brassica* and *Arabidopsis* may be advanced. One explanation is that the role of *MLPK*, *ARC1*, *Exo70A1*, and autophagy in SI is not universal among crucifers and that distinct SRK-mediated pollen rejection pathways may operate in different species. Another explanation is suggested by a comparison of the phenotypes produced by manipulating expression of *SRK* on the one hand, and of *ARC1*, *Exo70A1*, and genes required for autophagy on the other hand. While disruption of *SRK* expression causes complete breakdown of SI, down-regulation of *ARC1* or overexpression of *Exo70A1* in *Brassica* and loss of autophagy in transgenic self-incompatible *A. thaliana* plants only weaken, but do not abolish, the SI response (Abhinandan et al. 2022). These observations, together with the various cytological changes triggered by self-pollen, strongly suggest that robust inhibition of self-pollen is ensured by the operation of parallel pathways forming an SI signaling network (Fig. 5; Tantikanjana et al. 2010), some branches of which may be preferentially used in different species.

One signaling pathway impacted by self-pollination which appears to function in both *Brassica* and *A. thaliana* and may thus be applicable to other self-incompatible crucifers, is the FER-Rop-RBOH-mediated signaling pathway which controls ROS levels in the stigma. In self-incompatible *B. rapa*, cross-pollination causes a reduction in basal stigmatic ROS levels like that observed in *A. thaliana* compatible pollinations, while self-pollination activates ROS production and triggers an increase in ROS levels (Zhang et al. 2021). Subsequent analysis of *A. thaliana SRK-SCR* transformants showed that the SRK-SCR interaction results in recruitment of FER and activation of FER-mediated ROS production, thus causing rejection of self-pollen (Fig. 5; Huang et al. 2023). It is not known how this SRK-to-ROS pathway relates to other cellular responses that are triggered by self-pollen and if its outcome spreads within the papilla beyond the cytoplasmic region subtending the incompatible pollen grain. In *B. rapa*, half-stigma pollinations in which incompatible pollen was applied to one half of a stigma and compatible pollen was applied to the other half did show that ROS levels increased in the first half and decreased in the second half (Zhang et al. 2021). While this result was interpreted as evidence for a localized increase or decrease in ROS levels (Zhang et al. 2021), it should be noted that the half-stigma pollination assay does not, as claimed by the authors, mimic the situation in nature where the entire stigma, and even a single papilla, is challenged by a mixture of compatible and incompatible pollen grains. A more informative assay would use micromanipulators to apply a self-pollen grain and a nonself-pollen grain to the same papilla. Indeed, such dual-pollination assays were carried out on individual papillae as early as the 1980s. In *Brassica*, a papilla was shown to simultaneously inhibit a self-pollen grain and allow a nonself-pollen grain to hydrate and germinate, suggesting a highly localized SI response (Sarker et al. 1988). By contrast, more recent dual-pollination assays performed on individual papillae of self-incompatible *A. thaliana SRK-SCR* transformants showed that both self- and nonself-pollen were inhibited in most dual pollinations. This result suggests that the consequences of SI signaling are not restricted to the site of contact with self-pollen, but they rather diffuse rapidly

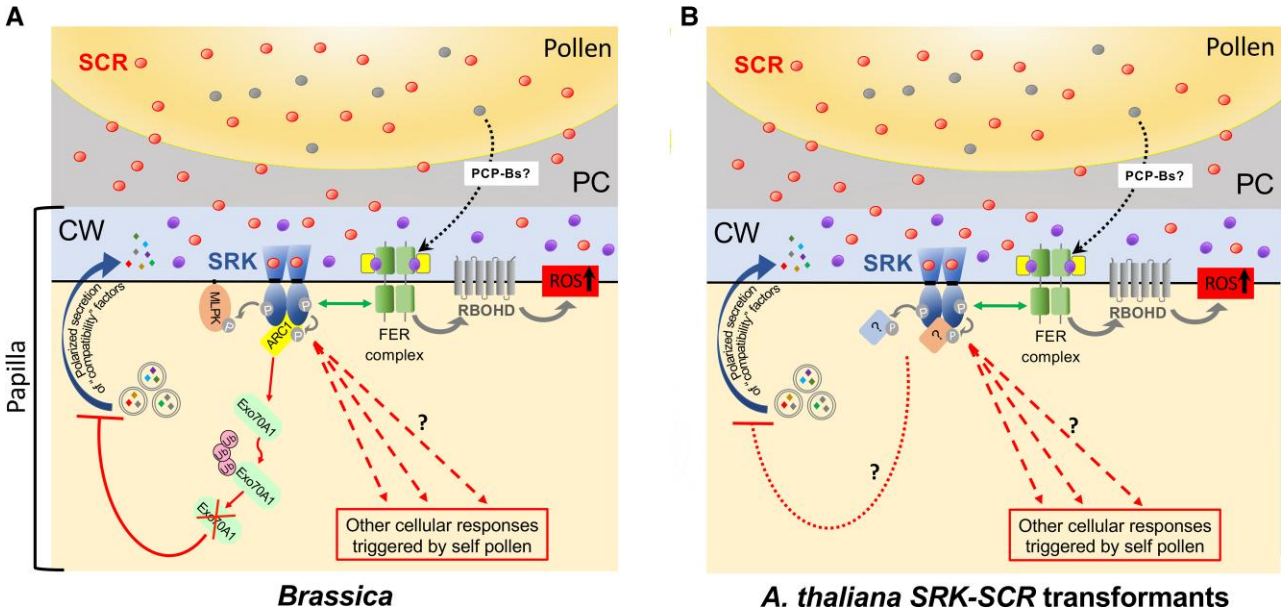

Figure 5. Molecular events underlying self-incompatible interactions at the papilla–pollen grain interface in *Brassica* and transgenic *A. thaliana SRK-SCR* plants. Naturally occurring self-incompatible plants such as *Brassica* plants are heterozygous at the *S* locus; thus, a stigmatic papilla expresses 2 different *SRK* variants and a pollen grain generally carries 2 SCR variants (except in the case of dominant-recessive *SCR* interactions as described in the text). For simplicity, only 1 SRK variant and its cognate SCR are shown in the *Brassica* diagram. On the other hand, transgenic *A. thaliana SRK-SCR* plants, which are typically designed to express one SI specificity, are hemizygous (primary transformants) or homozygous (transgenic progenies) for 1 *SRK-SCR* gene pair. **A** and **B)** The diagrams depict the interaction of SRK with its cognate SCR in *Brassica* and self-incompatible *A. thaliana SRK-SCR* transgenic plants, the resulting autophosphorylation of the receptor, subsequent phosphorylation of downstream substrates, and proposed SRK-mediated signaling pathways that lead to inhibition of self-pollen. Inhibition of polarized secretion of "compatibility" factors (curved arrow) and maintenance of high ROS levels by interaction (double-headed arrow) between SRK and the FER-ANJ-LLG1 complex (FER complex bound to the RALF33 peptide) are shown as major outcomes of SRK-mediated signaling. Several hypothetical pathways which together produce the various cellular responses triggered by self-pollen (see text) are shown as operating in parallel (dashed arrows with question marks), but intersecting pathways are also possible. How PCP-Bs impact the proposed SRK-mediated pathways is not known. **A)** In *Brassica*, MLPK, ARC1, and the ARC1-Exo70A1 pathway leading to degradation of ubiquinated Exo70A1 and inhibition of polarized secretion (blunt curved arrow) are shown. **B)** In *A. thaliana*, the downstream targets of SRK are not known (boxes with question marks) and the unknown mechanism by which polarized secretion is inhibited is indicated by the dashed blunt curved arrow. CW, cell wall; PC, pollen coat.

into other parts of the papilla (Iwano et al. 2015). While the latter result is consistent with the observation that self-pollen-induced actin depolymerization is observed throughout the papilla (Iwano et al. 2007), additional work is required to determine if the discrepancies between the two studies are real or if they are due to differences in the number of assays performed.

Cross-incompatibility between races, species, and genera

The most common form of cross-incompatibility is interspecific incompatibility, a post-pollination prezygotic reproductive barrier like SI which causes the pistil to reject pollen from other species (interspecific pollen), thus preventing gene flow between species and possibly contributing to speciation. In interspecific incompatibility, the site of pollen inhibition in the pistil, the mode of pollen rejection, and its regulation as a function of pistil development are identical to the rejection of self-pollen in self-incompatible plants (Kandasamy et al. 1994). As stated by Heslop-Harrison (1975), "the obvious distinction is to be seen in the effects: while interspecific systems preclude too remote a union, intraspecific SI systems prevent one that is too close." The similar manifestation of pollen rejection and the correlated evolution of SI and interspecific incompatibility (Roda and Hopkins 2019) have suggested a mechanistic overlap between the two pollination barriers. Indeed, it has been proposed that the rejection of interspecific pollen may be an inevitable pleiotropic consequence of SI (Roda and Hopkins 2019). In support of this notion, the most common form of interspecific incompatibility is the asymmetric reproductive barrier known as unilateral incompatibility (UI). UI is most often observed in reciprocal crosses between closely related self-incompatible and self-fertile species. In these crosses, the pistils of self-incompatible species reject pollen from SC species while the pistils of SC species accept pollen from self-incompatible species (Lewis and Crowe 1958). This so-called "SI × SC" rule has further led to the suggestion that a common pollen rejection pathway, and even a requirement for SRK, may be shared by these two incompatibility systems. The overlap is not perfect however, because UI has also been observed in crosses between

self-incompatible species, between SC species (Li et al. 2018), and more rarely between self-incompatible strains of one species.

An unusual case of intraspecific UI was observed in crosses between self-incompatible strains of *B. rapa* from Japan and Turkey. Contrary to the expectation that these strains would be cross-compatible because of the different *S* haplotypes they express, the stigmas of the Japanese line reject pollen from the Turkish line while the stigmas of the Turkish line accept pollen from the Japanese line (Takada et al. 2021). Interestingly, this UI system was found to be based on the activity of tightly linked *SRK*-like and *SCR*-like genes that were apparently produced by duplication of the *bona fide* *S* locus and subsequent translocation of the duplicated segment to a new genomic location. In this case, intraspecific UI is explained by the occurrence of stochastic mutations which inactivated the stigma *SRK*-like gene in Turkish strains and the pollen *SCR*-like gene in Japanese strains (Takada et al. 2021).

Several studies of the more common interspecific UI have discounted a role for SRK and some components of the SRK signaling pathway in interspecific pollen rejection. Reciprocal pollinations between self-incompatible *B. oleracea* and *A. thaliana* obeyed the SI × SC rule, but the stigmas of a *B. oleracea* strain carrying a kinase-deleted *SRK* was still able to reject *A. thaliana* pollen (Kandasamy et al. 1994). Similarly, analysis of 2 *B. rapa* strains that differed in their stigmas' ability to reject *B. oleracea* pollen found no evidence for the involvement of SRK or MLPK in interspecific incompatibility (Udagawa et al. 2010). Finally, a genome-wide association study involving a large collection of accessions that differ in their ability to reject pollen from distantly related crucifer species identified the stigma-specific *Stigmatic Privacy 1* (*SPR1*) gene which encodes a previously uncharacterized protein that acts in an SRK-independent manner (Fujii et al. 2019). Interestingly, *SPR1* may use a somewhat different pollen rejection pathway than that used by SI because it does not inhibit the hydration of interspecific pollen but rather blocks pollen germination and pollen tube entry into the stigma (Fujii et al. 2019).

In contrast to these examples of SRK-independent interspecific incompatibility, other studies did find evidence for the involvement of SRK-dependent pathways, thus pointing to the mechanistic complexity of interspecific pollen rejection. A genetic analysis of unilateral interspecific and intergeneric pollen rejection in the relatives of *A. thaliana* inferred that these systems share a common pollen rejection pathway with SI (Li et al. 2018). More recently, the SI × SC type of UI was found to be based on SRK-dependent activation of FER-controlled ROS production, this time triggered, not by SCR, but by a signal from heterospecific pollen (Huang et al. 2023). In this context, it should be noted that the conclusion that SI and interspecific incompatibility are coordinately gained or lost (Huang et al. 2023) does not agree with the previously reported uncoupling of the two incompatibility systems in *Brassica* and in transgenic *A. thaliana* (Kandasamy et al. 1994; Nasrallah et al. 2002) (Table 1). In any case, the nature of the proposed heterospecific pollen-derived signal, if it interacts with SRK or with an as-yet unidentified receptor, and if the same signal is produced by pollen donor species irrespective of their phylogenetic relationship to the recipient species are not known. Also not known is how the SRK-dependent FER-to-ROS pathway would apply to unilateral interspecific incompatibilities that do not conform to the SI × SC rule, especially those observed in SC × SC species pairs (Li et al. 2018).

Breakdown of SI: spontaneous mutations, evolutionary transitions to self-fertility, and experimental manipulation of SI

Spontaneous mutations and evolutionary transitions to self-fertility

In the face of varying selective pressures for outcrossing and selfing, SI has experienced recurring origin and loss and has occasionally been regained (Zhao et al. 2022). As a means of reproductive assurance when outcross pollen is limiting or when mates are scarce, evolutionary transitions to self-fertility have occurred frequently and are a major evolutionary trend in plants. Consequently, crucifer genera, like many other angiosperm genera, are generally comprised of self-incompatible and secondarily evolved SC species, and a predominantly self-incompatible species often includes self-fertile strains or cultivars. These strains have been used to identify the genetic events that underlie transitions to self-fertility, first by classical genetic analysis and more recently by large-scale DNA sequencing of populations segregating for self-fertility or of self-fertile species or strains collected from the wild. Together, these analyses have identified loss-of-function mutations in *SRK*, *SCR*, or both which inactivate the *S* locus (Nasrallah et al. 1994; Boggs et al. 2009a; Guo et al. 2011; Tsuchimatsu et al. 2017) and other mutations that disrupt "modifier" loci unlinked to the S locus, some of which function in the regulation of S-locus gene expression (Nasrallah 1974; Nasrallah et al. 1992, 2002), while others have as yet unknown functions.

The evolutionary transition to self-fertility is best understood in *A. thaliana*. The fact that several *A. thaliana* accessions acquire the SI trait when transformed with functional *SRK-SCR* gene pairs indicates that the species descended from a self-incompatible ancestor. The *A. thaliana* *S* locus, whose genomic location was identified by comparison with the *A. lyrata* *S* locus (Kusaba et al. 2001), was initially analyzed in a small number of geographical accessions (Kusaba et al. 2001; Sherman-Broyles et al. 2007; Tang et al. 2007; Boggs et al. 2009a) and more recently in a comprehensive species-wide analysis of the entire *S*-locus region in 1,083 accessions by mining resequencing data and by targeted resequencing (Tsuchimatsu et al. 2017). Together, these studies found no evidence for the existence of functional *S* haplotypes in the species. The data demonstrate that *A. thaliana* has retained only 3 groups of divergent *S* haplotypes

Table 1. Discrepancies in the reported response of stigmas to heterospecific pollen

STIGMA PARENT[a]	POLLEN PARENT[b]				References
	A. thaliana	*A. lyrata* $S_{20}S_{20}$	*A. lyrata* $S_{13}S_{13}$	*Barbarea vulgaris* *Brassica oleracea* *Brassica rapa*	
1. **SC** *Arabidopsis thaliana*	C	C	C	C	Nasrallah et al. 2002
2. **SI** *A. thaliana* C24[AlS_{20}]	C	I	C		Nasrallah et al. 2002
3. **SI** *A. thaliana* [AhS_{13}]				I	Huang et al. 2023
4. **SI** *B. oleracea* S_6S_6	I				Kandasamy et al. 1994
5. **SI** *B. rapa* $S_{46}S_{46}$	I			I	Huang et al. 2023
6. **SC** *B. oleracea* $SRK_{Sf1}SRK_{Sf1}$	I				Nasrallah et al., 1994 Kandasamy et al. 1994
7. **SC** *B. rapa* $BrSRK^{\Delta TM}BrSRK^{\Delta TM}$				C	Huang et al. 2023

[a]The first column shows the plants from which stigmas used for pollination were derived: (1) self-compatible (SC) untransformed *A. thaliana;* (2) self-incompatible (SI) *A. thaliana* C24 plants carrying the *SRK-SCR* transgenes from the *A. lyrata* S_{20} haplotype (AlS_{20}); (3) SI *A. thaliana* (accession unknown) carrying *SRK-SCR* transgenes from the *A. halleri* S_{13} haplotype (AhS_{13}); (4) SI *B. oleracea* S_6S_6 homozygote; (5) SI *B. rapa* $S_{46}S_{46}$ homozygote; (6) SC *B. oleracea* homozygous for the defective SRK_{Sf1} gene which lacks the kinase domain; (7) SC *B. rapa* homozygous for the defective $BrSRK^{\Delta TM}$ gene which lacks the transmembrane domain. Note that the AlS_{13} and AlS_{20} designations replace, respectively, the *AlSa* and *AlSb* designations in Nasrallah et al. (2002) which predated the current numbering of *A. lyrata S* haplotypes.

[b]Columns 2-5 show the outcome of manual pollinations in which stigmas derived from plants in column 1 are pollinated with pollen derived from *A. thaliana, A. lyrata, Barbarea,* or *Brassica* strains. C: compatible pollination; I: incompatible pollination. Shaded boxes highlight discrepancies in the reported outcome of heterospecific pollinations of stigmas from transgenic *A. thaliana* plants that express SI (shaded boxes in rows 2 and 3) and from *Brassica* strains that are self-compatible due to defective *SRK* genes (shaded boxes in rows 6 and 7).

(designated *S* haplogroups) which share high sequence similarity with 3 *S* haplotypes found in extant accessions of self-incompatible *A. lyrata* and *A. halleri*, 2 species from which *A. thaliana* diverged approximately 5 million years ago. These haplogroups have been inactivated by loss-of-function mutations or partial deletions of their *SRK* or *SCR* genes or both. The fact that these ancient polymorphisms have been retained and that some *S* haplotypes still contain functional *SRK* genes is indicative of a recent switch to self-fertility in *A. thaliana*, consistent with estimates that the species transitioned to selfing between 150,000 and 1 million years ago (Payne and Alvarez 2018). Nevertheless, different accessions harbor nonfunctional *S* haplotypes in various states of decay which arose independently of one another (Fig. 4C). These haplotypes exhibit extensive restructuring and deletion of substantial segments of the locus, or they can result from interhaplogroup recombination events. These features are consistent with the expectation that the switch to self-fertility is associated with a reduced number of *S* haplotypes and with relaxation of selective pressures for maintaining the integrity of the *S* locus and its genes.

In addition to nonfunctional *S* haplotypes, *A. thaliana* accessions harbor cryptic mutations at SI "modifier" loci which seemingly arose stochastically in different populations of the species, as inferred from the distinct pollination phenotypes observed in *SRK-SCR* transformants of different accessions (Fig. 4C; Nasrallah et al. 2004). Due to this complex genetic architecture of selfing, it is difficult to determine which mutation was the primary mutation that caused loss of SI and which mutations were secondary mutations resulting from relaxed selective pressure on SI-related genes. Indeed, it is only for accessions which can be reverted to a strong and stable SI response, and therefore lack these cryptic mutations, that inactivation of the *S* locus may be deduced to have been the event that caused self-fertility (Fig. 4C; Boggs et al. 2009a). Because these accessions harbor distinct *S* haplotypes of independent origins (Fig. 4C), it is evident that the self-fertility of *A. thaliana* resulted from multiple independent events rather than from the selective sweep of a single nonfunctional *S* haplotype.

Mutagenesis of the SI response in transgenic self-incompatible *A. thaliana*

In *A. thaliana*, genetic screens have long been used to identify mutations that disrupt specific biological pathways and in principle, such screens could be performed in *SRK-SCR* transformants for identification of additional factors required for SI. However, standard protocols for seed mutagenesis and mutant screens require large numbers of seed, and their implementation in self-incompatible plants that do not set seed is challenging, whether in naturally self-incompatible *Brassica* spp. or in fully self-incompatible transgenic *A. thaliana* plants such as C24[*SRK-SCR*] transformants. By contrast, Col-0[*SRK-SCR*] transformants, which express transient SI produce large numbers of seed due to loss of SI in older stigmas, are suitable for standard mutagenesis of the SI response. Moreover, an advantage of mutant screens in Col-0[*SRK-SCR*] plants is that they can identify mutations that abolish the SI response in young stigmas (using labor-intensive screening by manual self-pollination in the young floral buds of thousands of plants) (Strickler et al. 2013) as well as mutations

Table 2. Pollen-stigma interactions in *Brassica* and *Arabidopsis* spp.: some reported differences

Process	Brassica	Arabidopsis	References[a]
Genetic ablation of papillae in *SLGpr:DT-A* transgenic plants	*B. oleracea:* Stunted and biochemically inactive papillae. Self-sterile and cross-sterile in reciprocal pollinations to wild type	*A. thaliana* C24 plants: Stunted and biochemically inactive papillae. Self-sterile, stigma and pollen functional in reciprocal pollinations to wild type	Kandasamy et al., 1993 (B) Thorsness et al. 1993 (A)
• ARC1 and Exo70A1; identified in yeast 2-hybrid screens • MLPK; identified by genetic analysis of a self-fertile strain	Required for SI in • *B. napus*: weakening of SI by down-regulation of *ARC1* or overexpression of Exo70A1 • *B. rapa:* complete breakdown of SI in most, but not all, plants homozygous for an *MLPK* loss-of-function mutation	Not required for SI in *A. thaliana* C24[*SRK-SCR*] plants: • No functional *ARC1* gene in the *A. thaliana* genome • No disruption of SI in Exo70A1 overexpressers or in plants carrying a loss-of-function mutation in the putative *MLPK* ortholog	Abhinandan et al. 2022 (B) Kitashiba et al. 2011 (A) Rozier et al. 2020 (A)
Activation of autophagy in the SI response; proposed to function in clearing compatibility factors to the vacuole for degradation	Autophagosomes not observed	In *A. thaliana SRK-SCR* transformants and in *A. lyrata:* autophagosomes are observed and loss of autophagy causes weakening of SI	Abhinandan et al. 2022 (A/B)

[a](A) and (B) indicate that the reference describes work done in *Arabidopsis* and *Brassica*, respectively.

that enhance the SI response (using simple screens for low or no seed set) (Tantikanjana et al. 2009).

Indeed, the most informative mutation recovered in a screen of mutagenized Col-0[SRK_{20}-SCR_{20}] plants is a recessive pleiotropic mutation that simultaneously enhances the SI response in papillae and increases pistil elongation causing the protrusion of stigmas above the anthers (stigma exsertion) (Tantikanjana et al. 2009). The stigma exsertion mutant phenotype requires a catalytically active SRK but does not require SCR, indicating that SRK has an SCR-independent role in pistil elongation. Further analysis revealed the involvement of AUXIN RESPONSE FACTOR3 (ARF3), which is expressed in the stylar vasculature and is implicated in the regulation of auxin signaling in the pistil. ARF3 acts noncell-autonomously from its location in the style to enhance SI and simultaneously down-regulate auxin responses in papillae (Tantikanjana and Nasrallah 2012). A possible explanation for these effects is that ARF3 controls the production of a mobile signal that negatively regulates SI in papillae. The involvement of ARF3 suggests that auxin would enhance pollen tube growth at the stigma surface while suppression of auxin signaling would promote, or be required for, rejection of self-pollen. While this hypothesis remains to be tested, the analysis has uncovered a mechanistic link between the SI and pistil developmental pathways. Such a link is supported by the frequent association of SI with adaptations in floral architecture, including stigma exsertion, which reduce the chance of self-pollination while increasing the likelihood of cross pollen receipt. The pleiotropic role of SRK in SI and stigma exsertion shows how selection can act on one trait and simultaneously affect the other trait, and thus cause the rapid changes in floral architecture that can occur after loss of SI during evolutionary switches from out-crossing to self-fertility (Foxe et al. 2009).

Breaking the SI barrier for pollination control in breeding programs

SI-based schemes are used in crucifer crop plants to produce desirable F1 hybrid cultivars and to generate hybrid seeds on a large scale. These schemes typically use one or more self-incompatible inbreds as parental lines that must be maintained by using some method to break SI.

Several treatments have been traditionally use to break the SI barrier. One of the more effective treatments is spraying inflorescences with a 5% sodium chloride solution. It has been suggested that the breakdown of SI caused by this treatment is due to sodium chloride-induced changes in the stigma proteome (Yang et al. 2018). However, a simple explanation is that sodium chloride induces plasmolysis of papillae and that retraction of the protoplast leads to physical separation of the plasma membrane from the cell wall, thus preventing SRK from interacting with its cell wall-localized cognate SCR ligand (Fig. 3B; Rea and Nasrallah 2015).

Recent studies have suggested new strategies for breaking pollination barriers between and within species. One strategy adds to the number of available treatments for overcoming incompatible pollen rejection. It involves treating stigmas with compounds that reduce ROS to promote the growth of self- and heterospecific pollen, which in the latter case would enable wide crosses for introgression of desirable traits into elite cultivars (Huang et al. 2023). Another strategy is based on an unexpected result obtained by transgenic manipulation of *A. thaliana SRK-SCR* transformants. An attempt to generate constitutive SI by expressing SRK along with its cognate SCR in papillae produced the opposite outcome, namely complete breakdown of SI due to entrapment of the SRK in the ER (Tantikanjana and Nasrallah 2015). This phenomenon is known as *cis* inhibition, and in the case of SRK, only occurs

when the receptor is coexpressed with its cognate SCR (Tantikanjana and Nasrallah 2015). These results have suggested schemes for overcoming the challenges of SI-based breeding which could be easily incorporated into ongoing breeding programs for vegetable, root, and seed cruciferous crops. In these schemes, *cis-SCR* transgenes designed for expression of specific SCR variants in papillae would be used as SRK-off switches for controlled breakdown of SI (Tantikanjana and Nasrallah 2015). As a bonus, the SRK-off switch could also be used along with a counter-selection marker for selection against the *cis-SCR* transgene to turn the SI response off and on as required in mutant screens. This regulatory cassette would overcome the obstacles to standard mutagenesis posed by the lack of seed in self-incompatible plants and thus allow the full exploitation of *A. thaliana SRK-SCR* plants for genetic analysis of the SI pollen rejection pathway.

Outlook

The review has highlighted the considerable complexity of the molecular mechanisms that lead to acceptance or rejection of pollen at the pollen-stigma interface in crucifers. While much has been learned, several unanswered questions remain. Some of the outstanding issues regarding details of compatible and incompatible pollen-papilla interactions were touched upon in various sections of the review. A major challenge for the future is to integrate the available information and construct a comprehensive view of pollen acceptance and rejection. It is critical to understand how the various pathways that coexist within the papilla operate in parallel or in competition with one another, to identify the points at which they might intersect, and to explore their possible redundancy. A case in point is the SRK-dependent and SRK-independent pathways for rejection of heterospecific pollen which are superimposed on the very weak exine-mediated adhesion of heterospecific pollen to the papilla (Zinkl et al. 1999). Do these pathways intersect at some point? Are they parallel pathways that reflect a built-in redundancy to ensure robust rejection of heterospecific pollen for the all-important preservation of ovules and maintenance of species integrity? Or does the activation of each pathway depend on the phylogenetic distance between pollen and stigma parents? Clearly, it is no longer desirable to investigate one pathway in isolation from the others.

Another important issue is to understand the various pathways in the context of documented differences between crucifer genera, *Brassica* and *Arabidopsis* in particular, some of which are shown in Table 2. It is currently not known if some of the discrepancies observed among various studies are due to the use as sources of stigma and pollen of different combinations of species having different phylogenetic relationships. Nevertheless, more impactful differences have emerged. Thus, species differences regarding the roles of MLPK and of the ARC1-Exo70A1 pathway and its cellular consequences (Table 2), together with species differences in the transcriptomic profiles of stigmas pollinated with compatible or incompatible pollen (Abhinandan et al. 2022), might suggest that distinct *SRK*-mediated signaling pathways may operate in *Brassica* and *Arabidopsis*. Given the major role proposed for the ARC1-Exo70A1 pathway, resolving the species discrepancies regarding the role of this pathway is especially important. The role of ARC1 should also be evaluated in light of a recent report, should its results be confirmed. This report describes overexpression in *A. thaliana* of an *ARC1*-like gene from *Erigeron breviscapus*, a member of the Asteraceae which exhibit an SSI system unrelated to the crucifer *SRK-SCR* system (Chen et al. 2020). Stigmas that over-express this gene exhibit an SI-like phenotype in the absence of *SRK* and *SCR* genes: self-pollination results in rejection of pollen at the stigma surface while reciprocal pollinations with wild type produce profuse pollen tube growth. Be it as it may, resolving the discrepancies between crucifer species will require further assessment of the available data as well as analysis of additional crucifer species to determine which results, if any, are genus- or species-specific and which may be generalized across crucifers.

Most challenging are evolutionary questions related to the diversification of the SI recognition repertoire and the origin of SI systems and their genes. A better understanding of the diversification process will require analysis of additional extant S haplotypes, structure-based rational modification of the SRK-SCR interaction interface for the ultimate goal of engineering a novel SI specificity, and assessment of engineered variants for binding affinity in vitro and allele specificity in planta. As for the origin of SI systems, it has long been proposed that these systems evolved from pathogen recognition pathways, and this hypothesis, or at least the notion of convergent evolution of SI and defense, has been supported by the similar nature of some of the genes that underlie the two recognition systems (e.g. the defensin-like SCR in crucifers) (Nasrallah 2005). While a definitive answer to this question is unlikely, future studies should, at the very least, seek to assess how many mechanistically different SI systems have evolved in angiosperms. This endeavor will require analysis of SI in many more plant families, a task that is made difficult by the extensive polymorphisms of SI specificity genes and the highly rearranged loci in which they reside. While the effort is daunting, resolving these most intriguing evolutionary questions will have profound implications for our understanding of the coevolution of interacting partners in a variety of self-/nonself-discrimination systems. It will also be crucial for deciphering the complex mechanisms that angiosperms have evolved to enforce outcrossing and ensure the genetic diversity required for their long-term fitness while avoiding costly interspecific hybridization for the maintenance of species integrity.

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References

Abhinandan K, Sankaranaraynan S, Macgregor S, Goring DR, Samuel MA. Cell-cell signaling during the Brassicaceae self-incompatibility response. Trends Plant Sci. 2022:**27**(5):472–487. https://doi.org/10.1016/j.tplants.2021.10.011

Bateman AJ. Self-incompatibility systems in angiosperms. III. Cruciferae. Heredity. 1955:**9**(1):53–68. https://doi.org/10.1038/hdy.1955.2

Boggs NA, Dwyer KG, Nasrallah JB, Nasrallah ME. In vivo detection of residues required for ligand-selective activation of the S-locus receptor in Arabidopsis. Curr Biol. 2009a:**19**(9):786–791. https://doi.org/10.1016/j.cub.2009.03.037

Boggs NA, Dwyer KG, Shah P, McCulloch AA, Nasrallah ME, Nasrallah JB. Expression of distinct self-incompatibility specificities in *Arabidopsis thaliana*. Genetics. 2009b:**182**(4):1313–1321. https://doi.org/10.1534/genetics.109.102442

Boggs NA, Nasrallah JB, Nasrallah ME. Independent S-locus mutations caused self-fertility in *Arabidopsis thaliana*. PLoS Genet. 2009c:**5**(3):e1000426. https://doi.org/10.1371/journal.pgen.1000426

Bosch M, Wang L. Pollen-stigma interactions in Brassicaceae: complex communication events regulating pollen hydration. J Exp Bot. 2020:**71**(9):2465–2468. https://doi.org/10.1093/jxb/eraa117

Boyes DC, Nasrallah ME, Vrebalov J, Nasrallah JB. The self-incompatibility (*S*) haplotypes of *Brassica* encode highly divergent and rearranged sequences of ancient origin. Plant Cell. 1997:**9**:237–247. https://doi.org/10.1105/tpc.9.2.237

Cadavid LF, Powell AE, Nicotra ML, Moreno M, Buss LW. An invertebrate histocompatibility complex. Genetics. 2004:**167**(1):357–365. https://doi.org/10.1534/genetics.167.1.357

Cameron C, Geitmann A. Cell mechanics of pollen tube growth. Curr Opin Genet Dev. 2018:**51**:11–17. https://doi.org/10.1016/j.gde.2018.03.008

Casselman AL, Vrebalov J, Conner JA, Singhal A, Giovannoni J, Nasrallah ME, Nasrallah JB. Determining the physical limits of the *Brassica S* locus by recombinational analysis. Plant Cell. 2000:**12**(1):23–34. https://doi.org/10.1105/tpc.12.1.23

Chantreau M, Poux C, Lensink MF, Brysbaert G, Vekemans X, Castric V. Asymmetrical diversification of the receptor-ligand interaction controlling self-incompatibility in Arabidopsis. Elife. 2019:**8**:e50253. https://doi.org/10.7554/eLife.50253

Charlesworth D. How can two-gene models of self-incompatibility generate new specificities? Plant Cell. 2000:**12**(3):309–310. https://doi.org/10.1105/tpc.12.3.309

Charlesworth D. Why and how do Y chromosome stop recombining? J Evol Biol. 2023:**36**(3):632–636. https://doi.org/10.1111/jeb.14137

Chen M, Fan W, Hao B, Zhang W, Yan M, Zhao Y, Liang Y, Liu G, Lu Y, Zhang G, et al. *EbARC1*, an E3 ubiquitin ligase gene in *Erigeron breviscapus*, confers self-incompatibility in transgenic *Arabidopsis thaliana*. Int J Mol Sci. 2020:**21**(4):1458. https://doi.org/10.3390/ijms21041458

Cheung AY, Duan Q, Li C, James Liu MC, Wu HM. Pollen-pistil interactions: it takes two to tangle but a molecular cast of many to deliver. Curr Opin Plant Biol. 2022:**69**:102279. https://doi.org/10.1016/j.pbi.2022.102279

Chookajorn T, Kachroo A, Ripoll DR, Clark AG, Nasrallah JB. Specificity determinants and diversification of the *Brassica* self-incompatibility pollen ligand. Proc Natl Acad Sci U S A. 2004:**101**(4):911–917. https://doi.org/10.1073/pnas.2637116100

Conner JA, Conner P, Nasrallah ME, Nasrallah JB. Comparative mapping of the *Brassica S* locus region and its homolog in Arabidopsis: implications for the evolution of mating systems in the Brassicaceae. Plant Cell. 1998:**10**(5):801–812. https://doi.org/10.1105/tpc.10.5.801

Darwin C. On the different forms of flowers on the same species. London (UK): Murray; 1877.

Dearnaley J, Daggard G. Expression of a polygalacturonase enzyme in germinating pollen of *Brassica napus*. Sex Plant Reprod. 2001:**13**(5):265–271. https://doi.org/10.1007/s004970000062

Dickinson HG. Dry stigmas, water, and self-incompatibility in *Brassica*. Sex Plant Reprod. 1995:**8**(1):1–10. https://doi.org/10.1007/BF00228756

Di Giorgio JA, Bienert GP, Ayub ND, Yaneff A, Barberini ML, Mecchia MA, Amodeo G, Soto GC, Muschietti JP. Pollen-specific aquaporins NIP4; 1 and NIP4; 2 are required for pollen development and pollination in *Arabidopsis thaliana*. Plant Cell. 2016:**28**(5):1053–1077. https://doi.org/10.1105/tpc.15.00776

Dixit R, Nasrallah ME, Nasrallah JB. Post-transcriptional maturation of the S receptor kinase of *Brassica* correlates with co-expression of the S-locus glycoprotein in the stigmas of two *Brassica* strains and in transgenic tobacco plants. Plant Physiol. 2000:**124**(1):297–311. https://doi.org/10.1104/pp.124.1.297

Dong J, Kim ST, Lord EM. Plantacyanin plays a role in reproduction in Arabidopsis. Plant Physiol. 2005:**138**(2):778–789. https://doi.org/10.1104/pp.105.063388

Durand E, Chantreau M, Le Veve A, Stetsenko R, Dubin M, Genete M, Llaurens V, Poux C, Roux C, Billiard S, et al. Evolution of self-incompatibility in the Brassicaceae: lessons from a textbook example of natural selection. Evol Appl. 2020:**13**(6):1279–1297. https://doi.org/10.1111/eva.12933

Durand E, Méheust R, Soucaze M, Goubet PM, Gallina S, Poux C, Fobis-Loisy I, Guillon E, Gaude T, Sarazin A, et al. Dominance hierarchy arising from the evolution of a complex small RNA regulatory network. Science. 2014:**346**(6214):1200–1205. https://doi.org/10.1126/science.1259442

Dwyer KG, Balent MA, Nasrallah JB, Nasrallah ME. DNA Sequences of self-incompatibility genes from *Brassica campestris* and *B. oleracea*: polymorphism predating speciation. Plant Mol. Biol. 1991:**16**(3):481–486. https://doi.org/10.1007/BF00024000

Dwyer KG, Berger MT, Ahmed R, Hritzo MK, McCulloch AA, Price MJ, Serniak NJ, Walsh LT, Nasrallah JB, Nasrallah ME. Molecular characterization and evolution of self-incompatibility genes in *A. thaliana:* the case of the *Sc* haplotype. Genetics. 2013:**193**(3):985–994. https://doi.org/10.1534/genetics.112.146787

Edlund AF, Swanson R, Preuss D. Pollen and stigma structure and function: the role of diversity in pollination. Plant Cell. 2004:**16-**(Suppl):S84–S97. https://doi.org/10.1105/tpc.015800

Ferris PJ, Goodenough UW. The mating-type locus of *Chlamydomonas reinhardtii* contains highly rearranged DNA sequences. Cell. 1994:**76**(6):1135–1145. https://doi.org/10.1016/0092-8674(94)90389-1

Foxe JP, Slotte T, Stahl EA, Neuffer B, Hurka H, Wright SI. Recent speciation associated with the evolution of selfing in Capsella. Proc Natl Acad Sci U S A. 2009:**106**(13):5241–5245. https://doi.org/10.1073/pnas.0807679106

Franklin-Tong VE. Self-incompatibility in flowering plants. Evolution, diversity, and mechanisms. Berlin, Heidelberg: Springer-Verlag; 2008.

Fujii S, Takayama S. Multilayered dominance hierarchy in plant self-incompatibility. Plant Reprod. 2018:**31**(1):15–19. https://doi.org/10.1007/s00497-017-0319-9

Fujii S, Tsuchimatsu T, Kimura Y, Ishida S, Tangpranomkorn S, Shimosato-Asano H, Iwano M, Furukawa S, Itoyama W, Wada Y. A stigmatic gene confers interspecies incompatibility in the Brassicaceae. Nat Plants. 2019:**5**(7):731–741. https://doi.org/10.1038/s41477-019-0444-6

Giranton JL, Dumas C, Cock JM, Gaude T. The integral membrane S-locus receptor kinase of Brassica has serine/threonine kinase activity in a membranous environment and spontaneously forms oligomers in planta. Proc Natl Acad Sci U S A. 2000:**97**(7):3759–3764. https://doi.org/10.1073/pnas.97.7.3759

Goubet PM, Bergès H, Bellec A, Prat E, Helmstetter N, Mangenot S, Gallina S, Holl AC, Fobis-Loisy I, Vekemans X, et al. Contrasted patterns of molecular evolution in dominant and recessive self-incompatibility haplotypes in Arabidopsis. PLoS Genet. 2012:**8**(3): e1002495. https://doi.org/10.1371/journal.pgen.1002495

Guo YL, Zhao X, Lanz C, Weigel D. Evolution of the *S*-locus region in Arabidopsis relatives. Plant Physiol. 2011:**157**(2):937–946. https://doi.org/10.1104/pp.111.174912

Hamilton ES, Jensen GS, Maksaev G, Katims A, Sherp AM, Haswell ES. Mechanosensitive channel MSL8 regulates osmotic forces during pollen hydration and germination. Science. 2015:**350**(6259):438–441. https://doi.org/10.1126/science.aac6014

Hartmann FE, Duhamel M, Carpentier F, Hood ME, Foulongne-Oriol M, Silar P, Malagnac F, Grognet P, Giraud T. Recombination suppression and evolutionary strata around mating-type loci in fungi: documenting patterns and understanding evolutionary and mechanistic causes. New Phytol. 2020:**229**(5):2470–2491. https://doi.org/10.1111/nph.17039

Heslop-Harrison J. Incompatibility and the pollen-stigma interaction. Annu Rev Plant Physiol. 1975:**26**(1):403–425. https://doi.org/10.1146/annurev.pp.26.060175.002155

Hiscock SJ, Allen AM. Diverse cell signalling pathways regulate pollen-stigma interactions: the search for consensus. New Phytol. 2008: **179**(2):286–317. https://doi.org/10.1111/j.1469-8137.2008.02457.x

Huang J, Yang L, Yang L, Wu X, Cui X, Zhang L, Hui J, Zhao Y, Yang H, Liu S, et al. Stigma receptors control intraspecies and interspecies barriers in Brassicaceae. Nature. 2023:**614**(7947):303–308. https://doi.org/10.1038/s41586-022-05640-x

Iwano M, Igarashi M, Tarutani Y, Kaothien-Nakayama P, Nakayama H, Moriyama H, Yakabe R, Entani T, Shimosato-Asano H, Ueki M, et al. A pollen coat-inducible autoinhibited Ca2+-ATPase expressed in stigmatic papilla cells is required for compatible pollination in the Brassicaceae. Plant Cell. 2014:**26**(2):636–6c49. https://doi.org/10.1105/tpc.113.121350

Iwano M, Ito K, Fujii S, Kakita M, Asano-Shimosato H, Igarashi M, Kaothien-Nakayama P, Entani T, Kanatani A, Takehisa M, et al. Calcium signalling mediates self-incompatibility response in the Brassicaceae. Nat Plants. 2015:**1**(9):15128. https://doi.org/10.1038/nplants.2015.128

Iwano M, Shiba H, Funato M, Shimosato H, Takayama S, Isogai A. Immunohistochemical studies on translocation of pollen S-haplotype determinant in self-incompatibility of *Brassica rapa*. Plant Cell Physiol. 2003:**44**(4):428–346. https://doi.org/10.1093/pcp/pcg056

Iwano M, Shiba H, Matoba K, Miwa T, Funato M, Entani T, Nakayama P, Shimosato H, Takaoka A, Isogai A, et al. Actin dynamics in papilla cells of *Brassica rapa* during self- and cross-pollination. Plant Physiol. 2007:**144**(1):72–81. https://doi.org/10.1104/pp.106.095273

Johnson MA, Harper JF, Palanivelu R. A fruitful journey: pollen tube navigation from germination to fertilization. Annu Rev Plant Biol. 2019:**70**(1):809–837. https://doi.org/10.1146/annurev-arplant-050718-100133

Kachroo A, Schopfer CR, Nasrallah ME, Nasrallah JB. Allele-specific receptor-ligand interactions in *Brassica* self-incompatibility. Science. 2001:**293**(5536):1824–1826. https://doi.org/10.1126/science.1062509

Kakita M, Murase K, Iwano M, Matsumoto T, Watanabe M, Shiba H, Isogai A, Takayama S. Two distinct forms of M-locus protein kinase localize to the plasma membrane and interact directly with S-locus receptor kinase to transduce self-incompatibility signaling in *Brassica rapa*. Plant Cell. 2007:**19**(12):3961–3973. https://doi.org/10.1105/tpc.106.049999

Kandasamy MK, Nasrallah JB, Nasrallah ME. Pollen-pistil interactions and developmental regulation of pollen tube growth in *Arabidopsis*. Development. 1994:**120**(12):3405–3418. https://doi.org/10.1242/dev.120.12.3405

Kandasamy MK, Paolillo DJ, Faraday CD, Nasrallah JB, Nasrallah ME. The S-locus specific glycoproteins of Brassica accumulate in the cell wall of developing stigma papillae. Dev Biol. 1989:**134**(2):462–472. https://doi.org/10.1016/0012-1606(89)90119-X

Kandasamy MK, Thorsness MK, Rundle SJ, Goldberg ML, Nasrallah JB, Nasrallah ME. Ablation of papillar cell function in *Brassica* flowers results in the loss of stigma receptivity to pollination. Plant Cell. 1993:**5**(3):263–275. https://doi.org/10.2307/3869594

Kim S, Mollet JC, Dong J, Zhang K, Park SY, Lord EM. Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. Proc Natl Acad Sci U S A. 2003:**100**(26):16125–16130. https://doi.org/10.1073/pnas.2533800100

Kitashiba H, Liu P, Nishio T, Nasrallah JB, Nasrallah ME. Functional test of *Brassica* self-incompatibility modifiers in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A. 2011:**108**(44):18173–18178. https://doi.org/10.1073/pnas.1115283108

Kroh M. Reaction of pollen after transfer from one stigma to another (contribution of the character of the incompatibility mechanism in Cruciferae). Züchter/Genet Breed Res. 1966:**36**:185–189. https://doi.org/10.1007/BF02394157

Kusaba M, Dwyer KG, Hendershot J, Vrebalov J, Nasrallah JB, Nasrallah ME. Self-incompatibility in the genus Arabidopsis: characterization of the *S* locus in the outcrossing *A. lyrata* and its autogamous relative *A. thaliana*. Plant Cell. 2001:**13**(3):627–643. https://doi.org/10.1105/tpc.13.3.627

Kusaba M, Tung C-W, Nasrallah ME, Nasrallah JB. Monoallelic expression and dominance interactions in anthers of self-incompatible *Arabidopsis lyrata*. Plant Physiol. 2002:**128**(1):17–20. https://doi.org/10.1104/pp.010790

Lawrence MJ. Population genetics of the homomorphic self-incompatibility polymorphisms in flowering plants. Ann Bot. 2000:**85**:221–226. 10.1006/anbo.1999.1044

Leroux C, Bouton S, Kiefer-Meyer MC, Fabrice TN, Mareck A, Guénin S, Fournet F, Ringli C, Pelloux J, Driouich A. PECTIN METHYLESTERASE is involved in Arabidopsis pollen grain germination. Plant Physiol. 2015:**167**(2):367–380. https://doi.org/10.1104/pp.114.250928

Lewis D. Serological reactions of pollen incompatibility substances. Proc R Soc Lond B Biol Sci. 1952:**140**(898):127–135. https://doi.org/10.1098/rspb.1952.0049

Lewis D, Crowe LK. Unilateral incompatibility in flowering plants. Heredity. 1958:**12**(2):233–256. https://doi.org/10.1038/hdy.1958.26

Li DD, Guan H, Li F, Liu C Z, Dong YX, Zhang XS, Gao XQ. *Arabidopsis* shaker pollen inward K^+ channel SPIK functions in SnRK1 complex-regulated pollen hydration on the stigma. J Integr Plant Biol. 2017:**59**(9):604–611. https://doi.org/10.1111/jipb.12563

Li L, Liu B, Deng X, Zhao H, Li H, Xing S, Fetzen DD, Li M, Nasrallah ME, Nasrallah JB, et al. Evolution of interspecies unilateral incompatibility in the relatives of *Arabidopsis thaliana*. Mol Ecol. 2018: **27**(12):2742–2753. https://doi.org/10.1111/mec.14707

Liu C, Shen L, Xiao Y, Vyshedsky D, Peng C, Sun X, Liu Z, Cheng L, Zhang H, Han Z, et al. Pollen PCP-B peptides unlock a stigma peptide-receptor kinase gating mechanism for pollination. Science. 2021:**372**(6538):171–175. https://doi.org/10.1126/science.abc6107

Ma R, Han Z, Hu Z, Lin G, Gong L, Zhang H, Nasrallah JB, Chai J. Structural basis for specific self-incompatibility response in *Brassica*. Cell Res. 2016:**26**(12):1320–1329. https://doi.org/10.1038/cr.2016.129

McInnis SM, Desikan R, Hancock JT, Hiscock SJ. Production of reactive oxygen species and reactive nitrogen species by angiosperm stigmas and pollen: potential signalling crosstalk? New Phytol. 2006:**172**(2):221–228. https://doi.org/10.1111/j.1469-8137.2006.01875.x

Murase K, Moriwaki Y, Mori T, Liu X, Masaka C, Takada Y, Maesaki R, Mishima M, Fujii S, Hirano Y, et al. Mechanism of self/nonself-discrimination in Brassica self-incompatibility. Nat Commun. 2020:**11**(1):4916. https://doi.org/10.1038/s41467-020-18698-w

Naithani S, Chookajorn T, Ripoll DR, Nasrallah JB. Structural modules for receptor dimerization in the S-locus receptor kinase extracellular domain. Proc Natl Acad Sci U S A. 2007:**104**(29):12211–12216. https://doi.org/10.1073/pnas.0705186104

Nasrallah ME. Genetic control of quantitative variation in self-incompatibility proteins detected by immunodiffusion. Genetics. 1974:**76**(1):45–50. https://doi.org/10.1093/genetics/76.1.45

Nasrallah JB. Cell-cell signaling in the self-incompatibility response. Curr Opin Plant Biol. 2000:**3**(5):368–373. https://doi.org/10.1016/S1369-5266(00)00098-4

Nasrallah JB. Recognition and rejection of self in plant self-incompatibility: comparisons to animal histocompatibility. Trends Immunol. 2005:**26**(8):412–418. https://doi.org/10.1016/j.it.2005.06.005

Nasrallah JB. Evolution of the *Brassica* self-incompatibility locus: A look into *S*-locus gene polymorphisms. Proc Natl Acad Sci. 1997:**94**(18):9516–9519. http://dx.doi.org/10.1073/pnas.94.18.9516

Nasrallah ME, Kandasamy MK, Nasrallah JB. A genetically defined trans-acting locus regulates S-locus function in *Brassica*. Plant J. 1992:**2**(4):497–506. https://doi.org/10.1111/j.1365-313X.1992.00497.x

Nasrallah JB, Kao T-H, Chen C-H, Goldberg ML, Nasrallah ME. Amino acid sequences of glycoproteins encoded by three alleles at the S locus of *Brassica oleracea*. Nature. 1987:**326**(6113):617–619. https://doi.org/10.1038/326617a0

Nasrallah ME, Liu P, Nasrallah JB. Generation of self-incompatible *Arabidopsis thaliana* by transfer of two S locus genes from *A. lyrata*. Science. 2002:**297**(5579):247–249. https://doi.org/10.1126/science.1072205

Nasrallah ME, Liu P, Sherman-Brolyes S, Boggs NA, Nasrallah JB. Natural variation in expression of self-incompatibility in *Arabidopsis thaliana*: implications for the evolution of selfing. Proc Natl Acad Sci U S A. 2004:**101**(45):16070–16074. https://doi.org/10.1073/pnas.0406970101

Nasrallah JB, Nasrallah ME. Pollen-stigma signaling in the sporophytic self-incompatibility response. Plant Cell. 1993:**5**(10):1325–1335. https://doi.org/10.2307/3869785

Nasrallah JB, Nasrallah ME. Robust self-incompatibility in the absence of a functional ARC1 in *Arabidopsis thaliana*. Plant Cell. 2014:**26**(10):3838–3841. https://doi.org/10.1105/tpc.114.129387

Nasrallah JB. Plant mating systems: self-incompatibility and evolutionary transitions to self-fertility in the mustard family. Curr Opin Genet Dev. 2017:**47**:54–56. http://dx.doi.org/10.1016/j.gde.2017.08.005

Nasrallah JB, Rundle SJ, Nasrallah ME. Genetic evidence for the requirement of the Brassica S-locus receptor protein kinase gene in the pollen stigma interaction of self-incompatibility. Plant J. 1994:**5**(3):373–384. https://doi.org/10.1111/j.1365-313X.1994.00373.x

Nasrallah ME, Wallace DH. Immunochemical detection of antigens in self-incompatibility genotypes of cabbage. Nature. 1967:**213**(5077):700–701. https://doi.org/10.1038/213700a0

Nasrallah ME, Yogeeswaran Y, Snyder S, Nasrallah JB. Arabidopsis species hybrids in the study of species differences and evolution of amphiploidy in plants. Plant Phys. 2000:**124**(4):1605–1614. https://doi.org/10.1104/pp.124.4.1605

Ohata M, Takada Y, Sato Y, Okamoto T, Murase K, Takayama S, Suzuki G, Watanabe M. MLPK function is not required for self-incompatibility in the S^{29} haplotype of *Brassica rapa* L. Plant Reprod. 2023. https://doi.org/10.1007/s00497-023-00463-w

Payne BL, Alvarez-Ponce D. Higher rates of protein evolution in the self-fertilizing plant *Arabidopsis thaliana* than in the out-crossers *Arabidopsis lyrata* and *Arabidopsis halleri*. Genome Biol Evol. 2018:**10**(3):895–900. https://doi.org/10.1093/gbe/evy053

Quilichini TD, Grienenberger E, Douglas CJ. The biosynthesis, composition, and assembly of the outer pollen wall: a tough case to crack. Phytochem. 2015:**113**:170–182. https://doi.org/10.1016/j.phytochem.2014.05.002

Rea AC, Nasrallah JB. *In* vivo imaging of the S-locus receptor kinase, the female specificity determinant of self-incompatibility, in transgenic self-incompatible *Arabidopsis thaliana*. Ann Bot. 2015:**115**(5):789–805. https://doi.org/10.1093/aob/mcv008

Rejón JD, Delalande F, Schaeffer-Reiss C, Alché JD, Rodríguez-García MI, Van Dorsselaer A, Castro AJ. The pollen coat proteome: at the cutting edge of plant reproduction. Proteomes. 2016:**4**(1):5. https://doi.org/10.3390/proteomes4010005

Riglet L, Rozier F, Kodera C, Bovio S, Sechet J, Fobis-Loisy I, Gaude T. KATANIN-dependent mechanical properties of the stigmatic cell wall mediate the pollen tube path in Arabidopsis. Elife. 2020:**9**:e57282. https://doi.org/10.7554/eLife.57282

Roda F, Hopkins R. Correlated evolution of self and interspecific incompatibility across the range of a Texas wildflower. New Phytol. 2019:**221**(1):553–564. https://doi.org/10.1111/nph.15340

Rozier F, Riglet L, Kodera C, Bayle V, Durand E, Schnabel J, Gaude T, Fobis-Loisy I. Live-cell imaging of early events following pollen perception in self-incompatible *Arabidopsis thaliana*. J Exp Bot. 2020:**71**(9):2513–2526. 10.1093/jxb/eraa008

Sadowski EM, Hofmann CC. The largest amber-preserved flower revisited. Sci Rep. 2023:**13**(1):17. https://doi.org/10.1038/s41598-022-24549-z

Sainudiin R, Wong WSW, Yogeeswaran K, Nasrallah JB, Yang Z, Nielsen R. Detecting site-specific physicochemical selective pressures: applications to the class-I HLA of the human major histocompatibility complex and the SRK of the plant sporophytic self-incompatibility system. J Mol Evol. 2005:**60**(3):315–326. https://doi.org/10.1007/s00239-004-0153-1

Sarker RH, Elleman CJ, Dickinson HG. Control of pollen hydration in *Brassica* requires continued protein synthesis, and glycosylation in necessary for intraspecific incompatibility. Proc Natl Acad Sci U S A. 1988:**85**(12):4340–4344. https://doi.org/10.1073/pnas.85.12.4340

Sato K, Nishio T, Kimura R, Kusaba M, Suzuki T, Hatakeyama K, Ockendon DJ, Satta Y. Coevolution of the S-locus genes *SRK*, *SLG* and *SP11/SCR* in *Brassica oleracea* and *B. rapa*. Genetics. 2002:**162**(2):931–940. https://doi.org/10.1093/genetics/162.2.931

Schopfer CR, Nasrallah JB. Self-incompatibility: prospects for a novel peptide signaling molecule. Plant Physiol. 2000:**124**(3):935–940. https://doi.org/10.1104/pp.124.3.935

Schopfer CR, Nasrallah ME, Nasrallah JB. The male determinant of self-incompatibility in *Brassica*. Science. 1999:**286**(5445):1697–1700. https://doi.org/10.1126/science.286.5445.1697

Schweiger W, Steiner B, Vautrin S, Nussbaumer T, Siegwart G, Zamini M, Jungreithmeier F, Gratl V, Lemmens M, Mayer KF. Suppressed recombination and unique candidate genes in the divergent haplotype encoding Fhb1, a major Fusarium head blight resistance locus in wheat. Theor Appl Genet. 2016:**129**(8):1607–1623. https://doi.org/10.1007/s00122-016-2727-x

Sherman-Broyles S, Boggs N, Farkas A, Liu P, Vrebalov J, Nasrallah ME, Nasrallah JB. S locus genes and the evolution of self-fertility in *Arabidopsis thaliana*. Plant Cell. 2007:**19**(1):94–106. https://doi.org/10.1105/tpc.106.048199

Shiba H, Iwano M, Entani T, Ishimoto K, Shimosato H, Che FS, Satta Y, Ito A, Takada Y, Watanabe M, et al. The dominance of alleles controlling self-incompatibility in Brassica pollen is regulated at the RNA level. Plant Cell. 2002:**14**(2):491–504. https://doi.org/10.1105/tpc.010378

Shimosato H, Yokota N, Shiba H, Iwano M, Entani T, Che FS, Watanabe M, Isogai A, Takayama S. Characterization of the SP11/SCR high-affinity binding site involved in self/nonself recognition in Brassica self-incompatibility. Plant Cell. 2007:**19**(1):107–117. https://doi.org/10.1105/tpc.105.038869

Stein JC, Dixit R, Nasrallah ME, Nasrallah JB. SRK, the stigma-specific S locus receptor kinase of Brassica, is targeted to the plasma membrane in transgenic tobacco. Plant Cell. 1996:**8**:429–445. https://doi.org/10.1105/tpc.8.3.429

Stein JC, Howlett B, Boyes DC, Nasrallah ME, Nasrallah JB. Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc Natl Acad Sci U S A. 1991:**88**(19):8816–8820. https://doi.org/10.1073/pnas.88.19.8816

Strickler S, Tantikanjana T, Nasrallah JB. Regulation of the S-locus receptor kinase and self-incompatibility in *Arabidopsis thaliana*. G3. 2013:**3**(2):315–322. https://doi.org/10.1534/g3.112.004879

Takada Y, Mihara A, He Y, Xie H, Ozaki Y, Nishida H, Hong S, Lim YP, Takayama S, Suzuki G, et al. Genetic diversity of genes controlling unilateral incompatibility in Japanese cultivars of Chinese cabbage. Plants (Basel). 2021:**10**(11):2467. https://doi.org/10.3390/plants10112467

Takasaki T, Hatakeyama K, Suzuki G, Watanabe M, Isogai A, Hinata K. The S receptor kinase determines self-incompatibility in *Brassica stigma*. Nature. 2000:**403**(6772):913–916. https://doi.org/10.1038/35002628

Takayama S, Shimosato H, Shiba H, Funato M, Che FS, Watanabe M, Iwano M, Isogai A. Direct ligand-receptor complex interaction controls Brassica self-incompatibility. Nature. 2001:**413**(6855):534–538. https://doi.org/10.1038/35097104

Tang C, Toomajian C, Sherman-Broyles S, Plagnol V, Guo Y, Hu TT, Clark RM, Nasrallah JB, Weigel D, Nordborg M. The evolution of selfing in *Arabidopsis thaliana*. Science. 2007:**317**(5841):1070–1072. https://doi.org/10.1126/science.1143153

Tantikanjana T, Nasrallah JB. Non-cell-autonomous regulation of crucifer self-incompatibility by auxin response factor ARF3. Proc Natl Acad Sci U S A. 2012:**109**(47):19468–19473. https://doi.org/10.1073/pnas.1217343109

Tantikanjana T, Nasrallah JB. Ligand-mediated *cis*-inhibition of receptor signaling in the self-incompatibility response of the Brassicaceae. Plant Phys. 2015:**169**(2):1141–1154. https://doi.org/10.1104/pp.15.00572

Tantikanjana T, Nasrallah ME, Nasrallah JB. Complex networks of self-incompatibility signaling in the Brassicaceae. Curr Opin Plant Biol. 2010:**13**(5):520–526. https://doi.org/10.1016/j.pbi.2010.06.004

Tantikanjana T, Rivzi N, Nasrallah ME, Nasrallah JB. A dual role for the S-locus receptor kinase in self-incompatibility and pistil development revealed by an Arabidopsis *rdr6* mutation. Plant Cell. 2009:**21**(9):2642–2654. https://doi.org/10.1105/tpc.109.067801

Tarutani Y, Shiba H, Iwano M, Kakizaki T, Suzuki G, Watanabe M, Isogai A, Takayama S. Trans-acting small RNA determines dominance relationships in Brassica self-incompatibility. Nature. 2010:**466**(7309):983–986. https://doi.org/10.1038/nature09308

Thompson KF. Self-incompatibility in narrow-stem kale, *Brassica oleracea* var *acephala*. I. Demonstration of a sporophytic system. 1. Genetics. 1957:**55**:45–60.

Thorsness MK, Kandasamy MK, Nasrallah ME, Nasrallah JB. Genetic ablation of floral cells in Arabidopsis. Plant Cell. 1993:**5**(3):253–261. https://doi.org/10.2307/3869593

Tsuchimatsu T, Goubet PM, Gallina S, Holl AC, Fobis-Loisy I, Bergès H, Marande W, Prat E, Meng D, Long Q, et al. Patterns of polymorphism at the self-incompatibility locus in 1,083 *Arabidopsis thaliana* genomes. Mol Biol Evol. 2017:**34**(8):1878–1889. https://doi.org/10.1093/molbev/msx122

Udagawa H, Ishimaru Y, Li F, Sato Y, Kitashiba H, Nishio T. Genetic analysis of interspecific incompatibility in *Brassica rapa*. Theor Appl Genet. 2010:**121**(4):689–696. https://doi.org/10.1007/s00122-010-1340-7

Uyenoyama MK, Zhang Y, Newbigin E. On the origin of self-incompatibility haplotypes: transition through self-compatible intermediates. Genetics. 2001:**157**(4):1805–1817. https://doi.org/10.1093/genetics/157.4.1805

Wang L, Clarke LA, Eason RJ, Parker CC, Qi BX, Scott RJ, Doughty J. PCP-B class pollen coat proteins are key regulators of the hydration checkpoint in *Arabidopsis thaliana* pollen-stigma interactions. New Phytol. 2017:**213**(2):764–777. https://doi.org/10.1111/nph.14162

Wang R, Dobritsa AA. Exine and aperture patterns on the pollen surface: their formation and roles in plant reproduction. Annu Plant Rev. 2018:**1**:589–628. https://doi.org/10.1002/9781119312994.apr0625

Wang L, Lau YL, Fan L, Bosch M, Doughty J. Pollen coat proteomes of *Arabidopsis thaliana*, *Arabidopsis lyrata*, and *Brassica oleracea* reveal remarkable diversity of small cysteine-rich proteins at the pollen-stigma interface. Biomolecules. 2023:**13**(1):157. https://doi.org/10.3390/biom13010157

Windari EA, Ando M, Mizoguchi Y, Shimada H, Ohira K, Kagaya Y, Higashiyama T, Takayama S, Watanabe M, Suwabe K. Two aquaporins, SIP1; 1 and PIP1; 2, mediate water transport for pollen hydration in the *Arabidopsis* pistil. Plant Biotechnol (Tokyo). 2021:**38**(1):77–87. https://doi.org/10.5511/plantbiotechnology.20.1207a

Wolters-Arts M, Lush WM, Mariani C. Lipids are required for directional pollen-tube growth. Nature. 1998:**392**(6678):818–821. https://doi.org/10.1038/33929

Yamamoto M, Kitashiba H, Nishio T. Generation of Arabidopsis thaliana transformants showing the self-recognition activity of *Brassica rapa*. Plant J. 2022:**111**(2):496–507. https://doi.org/10.1111/tpj.15811

Yamamoto M, Tantikanjana T, Nishio T, Nasrallah ME, Nasrallah JB. Site-specific N-glycosylation of the S-locus receptor kinase and its role in the self-incompatibility response of the Brassicaceae. Plant Cell. 2014:**26**(12):4749–4762. https://doi.org/10.1105/tpc.114.131987

Yang Y, Liu Z, Zhang T, Zhou G, Duan Z, Li B, Dou S, Liang X, Tu J, Shen J, et al. Mechanism of salt-induced self-compatibility dissected by comparative proteomic analysis in *Brassica napus* L. Int J Mol Sci. 2018:**19**(6):1652. https://doi.org/10.3390/ijms19061652

Yasuda S, Kobayashi R, Ito T, Wada Y, Takayama S. Homology-based interactions between small RNAs and their targets control dominance hierarchy of male determinant alleles of self-incompatibility in *Arabidopsis lyrata*. Int J Mol Sci. 2021:**22**(13):6990. https://doi.org/10.3390/ijms22136990

Zhang L, Huang J, Su S, Wei X, Yang L, Zhao H, Yu J, Wang J, Hui J, Hao S, et al. FERONIA receptor kinase-regulated reactive oxygen species mediate self-incompatibility in *Brassica rapa*. Curr Biol. 2021:**31**(14):3004–3016.e4. https://doi.org/10.1016/j.cub.2021.04.060

Zhang T, Zhou G, Goring DR, Liang X, Macgregor S, Dai C, Wen J, Yi B, Shen J, Tu J, et al. Generation of transgenic self-incompatible *Arabidopsis thaliana* shows a genus-specific preference for self-incompatibility genes. Plants. 2019:**8**(12):570. https://doi.org/10.3390/plants8120570

Zhao H, Zhang Y, Zhang H, Song Y, Zhao F, Zhang Y, Zhu S, Zhang H, Zhou Z, Guo H, et al. Origin, loss, and regain of self-incompatibility in angiosperms. Plant Cell. 2022:**34**(1):579–596. https://doi.org/10.1093/plcell/koab266

Zinkl GM, Zwiebel BI, Grier DG, Preuss D. Pollen-stigma adhesion in Arabidopsis: a species-specific interaction mediated by lipophilic molecules in the pollen exine. Development. 1999:**126**(23):5431–5440. https://doi.org/10.1242/dev.126.23.5431