Minireview

Pollen recognition during the self-incompatibility response in plants

Simon J Hiscock

Address: School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK. E-mail: simon.hiscock@bristol.ac.uk

Published: 30 January 2002

Genome ***Biology*** 2002, **3(2)**:reviews1004.1–1004.6

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2002/3/2/reviews/1004

Abstract

Recent work has identified the elusive male (pollen) determinant that underlies self-incompatibility in *Brassica* (cabbage). The key pollen factor, recognized by the stigma of an incompatible plant, is a small cysteine-rich protein that interacts directly with the receptor domain of a stigma receptor serine-threonine kinase to initiate haplotype-specific pollen recognition and rejection.

Flowering plants, or angiosperms, are the most successful group of land plants and dominate the Earth's vegetation, with between 250,000 and 300,000 species. The remarkable success of flowering plants is a consequence of a suite of unique angiosperm features, most of which are found in the flower itself. A flower is an extremely efficient reproductive unit because it can promote male and female function at the same time, for example to optimize the benefits of insect pollination by ensuring that deposition of cross-pollen on stigmas and removal of self-pollen from anthers are accomplished during one insect visit. Co-sexuality, however, also increases the likelihood of self-pollination - but rarely does self-pollination necessarily lead to self-fertilization because many flowering plants (up to 70%) have genetic systems to ensure self-incompatibility (SI). Such systems allow the female pistil to recognize and then 'reject' self or self-related pollen, well before there is any risk of self-fertilization and its detrimental consequence, inbreeding depression (the converse of 'hybrid vigor'). Unique to flowering plants, SI systems can thus optimize the outbreeding potential of co-sexual flowers pollinated by insects or other animals.

Over the past 20 years SI has been the focus of intense molecular research and now, at last, a picture is emerging of how SI operates in certain plant groups. This article highlights recent work in *Brassica* (cabbage) species that has identified the interacting molecules in pollen and stigma that mediate pollen recognition and initiate the stigmatic SI response.

The genetics of self-incompatibility

SI is usually controlled by a single locus, *S* (for self-incompatibility), which has many alleles, denoted S_1, S_2, and so on. Indeed, the *S* locus is one of the most polymorphic loci known, comparable with the major histocompatibility complex (MHC) loci in mammals and mating-type loci in fungi and algae. When male pollen and female pistil share the same *S* allele, the pollen is identified as incompatible and rendered inactive (Figure 1). Classical genetic studies in the 1950s identified two distinct forms of SI, gametophytic (GSI) and sporophytic (SSI), which can be distinguished by the *S* phenotypic behavior of the pollen (Figure 1). The *S* phenotype of the pollen in GSI is determined by its own haploid genome, whereas in SSI the pollen *S* phenotype is determined by the diploid genome of its parent. This fundamental difference imposes serious constraints on the relative behavior of *S* alleles in the two SI systems. In GSI *S* alleles must be expressed co-dominantly in the pistil, but in SSI complex dominance relationships can exist between *S* alleles expressed in the pistil and also 'in' the pollen, because the pollen *S* phenotype is diploid. Thus, with functional GSI all individuals within a population will be heterozygous at *S*, as pollen could never fertilize a plant with the same *S* genotype as itself, but in an SSI population with *S* allelic dominance interactions a mix of *S* heterozygotes and *S* homozygotes is theoretically possible because recessive *S* alleles can be 'hidden' in pollen and/or stigma by dominant *S* alleles. Within particular angiosperm families

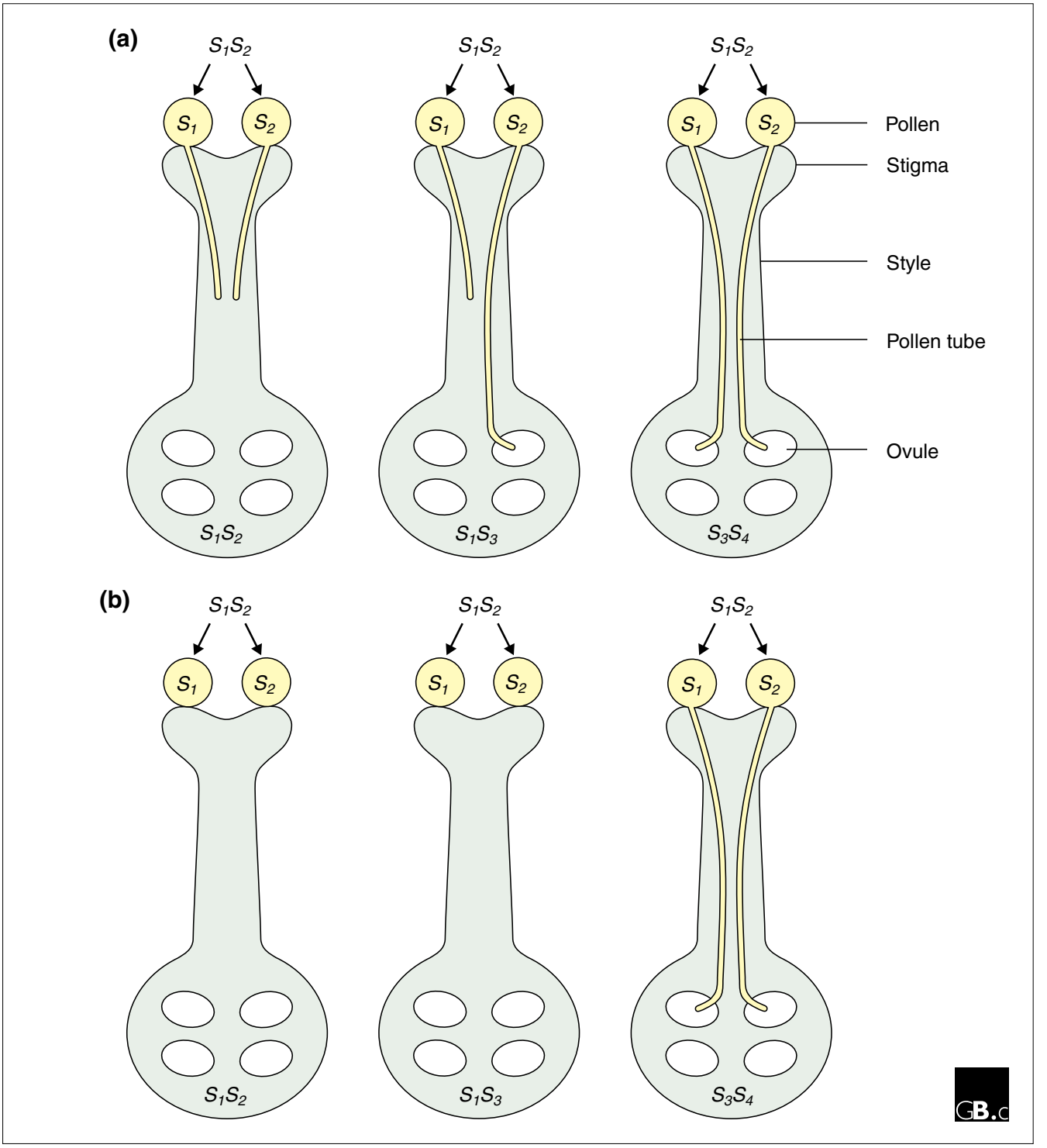

Figure 1
Genetic control of self-incompatibility (SI). When allelic forms (haplotypes) of the *S* locus are matched in pollen (male tissue) and pistil (female tissue) the pollen is rendered incompatible. The incompatibility response is mediated by a variety of molecular mechanisms in different species. **(a)** In GSI the SI phenotype of the pollen is determined by its own haploid genome, and the growing pollen tubes are usually inhibited in the style (the *S* alleles of the male and female parent plants and the haplotype of the pollen are indicated). GSI dictates that *S* alleles are expressed co-dominantly in the pistil. Note that 50% of pollen from an S_1S_2 individual is compatible with the S_1S_3 pistil because half the pollen grains express the S_1 allele and half the S_2 allele. **(b)** In SSI the SI phenotype of the pollen is determined by the diploid genome of its parent plant, so if *S* alleles are expressed co-dominantly 'in the pollen', 100% of the pollen from a S_1S_2 individual will be inhibited by an S_1S_3 pistil (in SSI, pollen is usually inhibited on the stigma). SSI is possible because pollen *S* alleles are expressed sporophytically in diploid cells of the anther tapetum that supplies the S proteins to the pollen coating.

SI is either gametophytic or sporophytic, so GSI is typically found in the Solanaceae (potato, tobacco, and tomato, for example), Rosaceae (apple, pear, and apricot) and Papaveraceae (poppy), whereas SSI is found in the Brassicaceae (cabbage family), Convolvulaceae (sweet potato and morning glory, for example) and Asteraceae (daisy family) [1].

Molecular diversity of self-incompatibility systems

The most striking finding made by molecular genetic analyses of SI during the 1980s and early 1990s was the extreme genetic diversity at the *S* locus [1]. Early studies of SSI in *Brassica* identified two stigma-expressed genes at the *S* locus, *S* locus glycoprotein (*SLG*) and *S* receptor serine-threonine kinase (*SRK*), and for a given *S* allele *SLG* was approximately 90% identical (at the amino-acid level) to the putative receptor domain of *SRK* [2]. Neither of these genes was found at the *S* locus of *Nicotiana*, which has GSI and a completely different gene, again pistil-specific in its expression, encoding a functional RNase [3]; correspondingly *S*-encoded self-incompatibility RNases (S-RNases) were not found at the *Brassica S* locus. These initial findings led to a predicted diversity in the molecular mechanisms of SI, a prediction that was duly confirmed when GSI in poppy was shown to operate through a novel Ca^{2+}-based signaling system distinct from the mechanism in either *Brassica* or *Nicotiana* [4].

Curiously, pistil-expressed (female) *S* genes have proved far more amenable to identification than pollen-expressed (male) *S* genes. Although *SLG*, *SRK* and S-RNase transcripts could be detected in the respective *Brassica* and *Nicotiana* pollens, their protein products could not. This suggests that, contrary to classical genetic theory [5], pollen (male) S and pistil (female) S proteins are encoded by different genes. Together with the finding of two female *S* genes at the *Brassica S* locus, this realization led to the view that *S* alleles were better described as *S* haplotypes, in accordance with the nomenclature for complex genetic loci.

The functional roles of S-RNases in GSI, and of SRK and SLG in SSI, were tested using transgenic loss-of-function and gain-of-function experiments. Stylar-expressed S-RNases were shown independently to be necessary and sufficient for *S*-haplotype-specific pollen rejection in *Petunia* [6] and *Nicotiana* [7]. More recent studies indicate that other pistil components, unlinked to *S*, are also necessary for S-RNase-mediated pollen rejection [8]. In *Brassica*, similar transgenic approaches demonstrated an essential role for SRK in *S*-haplotype-specific pollen rejection but, somewhat surprisingly, also revealed that SLG was dispensable for SI [9]. Nevertheless, expression of *SLG* did 'enhance' the strength of SRK-mediated pollen rejection (in terms of numbers of incompatible pollen grains failing to germinate), indicating that SLG may function as an accessory protein during the SSI response [9].

It is now clear that S-RNases regulate the female side of the SI reaction in a range of species outside the Solanaceae, for instance in the Rosaceae and Scrophulariaceae (such as *Antirrhinum*) [1]. S-RNases do not, however, regulate GSI in the Papaveraceae, where pollen rejection is triggered by a stigma-specific self-incompatibility (S-) glycoprotein (which has no known functional counterparts) that stimulates a Ca^{2+}-mediated signal transduction pathway in the pollen that terminates pollen tube development [10]. The finding of an alternative GSI pathway in poppy is important because it indicates that there is mechanistic diversity within genetically identical GSI systems, as well as between GSI and SSI. Interestingly, molecular diversity is also predicted within SSI, because recent evidence strongly suggests that *SRK* homologs do not mediate SSI in *Ipomoea* (Convolvulaceae) [11] or *Senecio* (Asteraceae) (S.H., unpublished observations). Clearly, SI systems are not homologous and must have arisen many times during angiosperm diversification.

Male and female self-incompatibility determinants in *Brassica*

In *Brassica* species it has long been known that the male determinant of SSI resides in the pollen coating [12]; this was clearly demonstrated by pollination bioassays in which extracted pollen coatings were 'swapped' between pollen grains to change their *S* phenotype [13]. Indeed, biochemical analyses of pollen-coat fractions capable of changing pollen *S* phenotype led to a prediction that the 'active' male protein was a small (less than 10 kDa) cysteine-rich protein [14,15]. It was by 'walking' between *SLG* and *SRK* within the *S* locus of *Brassica rapa* (syn. *campestris*), however, that the groups of Nasrallah [16] and Isogai [17] finally identified the long-elusive male determinant of SSI as a gene encoding a small cysteine-rich protein, expressed exclusively in anthers and displaying extreme *S*-allelic polymorphism. Somewhat ambiguously, allelic forms of pollen *S* were named *SCR* (for *S*-locus cysteine-rich) [16] and *SP11* (*S*-locus protein 11) [17]. Gain-of-function experiments showed conclusively that *SCR* determines *S*-haplotype-specific rejection of pollen in *B. rapa*: pollen from S_2S_2 homozygous plants transformed with an S_6-encoded *SCR6* cDNA was rejected by S_6S_6 plants but accepted by $S_{22}S_{22}$ plants [16]. This finding was supported by pollination assays in which recombinant S_9-SP11 applied to S_9S_9 and S_8S_8 stigmas induced rejection of cross or non-self pollen on S_9S_9 stigmas but not on S_8S_8 stigmas [17]. Gain-of-function experiments with plants transformed with S_9-*SP11* cDNA confirmed the pollination assay data for SP11 [18]. Interestingly, *SCR/SP11* is expressed in anther tissues both sporophytically (expressing the diploid genotype of the parent) and gametophytically (expressing the haploid genotype of the pollen) [17,18]. *In situ* hybridization pinpointed sporophytic expression to cells of the tapetum, a diploid tissue that nourishes developing pollen grains and provides components of the pollen coating, and gametophytic expression to microspores (the initial products of

male meiosis) and developing pollen grains and microspores [17]. This pattern of *SCR/SP11* expression easily explains how the parent plant determines the SI phenotype of the pollen, because all pollen grains receive tapetum-derived SCR/SP11 protein in their pollen coatings. So far, 22 *SCR/SP11* alleles have been identified in *Brassica* species, all of which encode small basic cysteine-rich proteins with a predicted molecular weight of less than 8 kDa [19].

With both male and female determinants of SSI identified in *Brassica*, elucidation of the biochemical mechanism of pollen recognition and rejection beckoned. Hypothetical models, based on analogies with receptor-kinase signaling systems in animals, predicted an *S*-haplotype-specific protein-protein interaction between SCR/SP11 (the putative ligand) and the extracellular receptor domain of SRK. This would lead to dimerization of activated SRKs and autophosphorylation on serines and threonines within the kinase domain (Figure 2) [20,21]. Recently, two papers, again from the groups of Nasrallah [22] and Isogai [23], have confirmed this model unambiguously. Kachroo *et al.* [22] used *in vitro* 'pull-down' assays to show haplotype-specific binding of tagged versions of the SRK_6 and SCR_6 proteins. A construct consisting of the receptor domain of SRK_6 carrying a carboxy-terminal FLAG epitope tag was expressed in tobacco leaves as a soluble, secreted glycoprotein and immobilized on FLAG affinity agarose. SCR_6 and SCR_{13}, expressed in bacteria as secreted proteins carrying a carboxy-terminal Myc-His_6 epitope tag, were then exposed at increasing concentrations to the SRK_6-FLAG agarose. Immunoblotting revealed that recombinant SRK_6 bound strongly to SCR_6-Myc-His_6 but only very poorly to SCR_{13}-Myc-His_6. In the reciprocal experiment, SCR_6-Myc-His_6 immobilized on nickel-agarose beads bound the recombinant SRK_6 and endogenous SRK_6 in microsomal extracts from S_6S_6 stigmas, but did not bind SRK_{13} from $S_{13}S_{13}$ microsomal extracts. These data clearly demonstrate that haplotype-specific binding between SCR and the receptor domain of SRK is the likely basis of haplotype-specific pollen rejection in *Brassica*.

This conclusion was reinforced by a subtly different set of protein-protein interaction experiments carried out by Takayama *et al.* [23], who used a synthetic form of S_8-SP11 produced chemically by solid-phase and solution methods. After initial confirmation that the synthetic S_8-SP11 was biologically active (pollination assays as described above were used), synthetic S_8-SP11 was radio-iodinated and exposed to microsomal stigmatic extracts from S_8S_8 and S_9S_9 plants. Immunoblots revealed that ^{125}I-labeled S_8-SP11 bound specifically to SRK_8 present in microsomal extracts of S_8S_8 stigmas but did not bind SRK_9 in microsomal fractions of S_9S_9 stigmas. The ^{125}I-labeled S_8-SP11/S_8S_8 stigma microsomal complex was then crosslinked and the components analyzed by gel electrophoresis and immunoblotting, which revealed not only the presence of SRK_8 but also the presence of SLG_8. As neither Takayama *et al.* [23] nor Kachroo *et al.* [22] could demonstrate any significant affinity between SP11/SCR and SLG, these data imply that SRK and SLG from the same haplotype associate during the SRK-SP11 haplotype-specific interaction. This finding may offer an explanation of why SLG apparently enhances *S*-haplotype-specific rejection of pollen but is itself a nonessential component of the SI rejection process [9]. If SLG is indeed an accessory protein stabilizing the receptor-ligand complex, what is the significance of SLG's strong affinity for PCP-A1, a small cysteine-rich pollen coat protein similar to SCR/SP11 but not linked to the *S* locus [14,15]? Perhaps PCP-A1, which has no affinity for SRK, acts as yet another accessory protein in stabilizing the receptor complex (Figure 2).

Importantly, Takayama *et al.* [23] went on to demonstrate that formation of the complex of S_8-SRK_8, SP11 and SLG_8 resulted in specific autophosphorylation of serine and threonine residues in the kinase domain of SRK_8. This confirmed the final prediction of the SSI model, namely that receptor dimerization leading to autophosphorylation follows haplotype-specific recognition and interaction between stigma SRK and its pollen ligand SCR/SP11 (Figure 2).

Cell signaling following pollen recognition

Now that the basis for pollen recognition during the SSI response has been established, the next phase of research must be to characterize the signal transduction pathway triggered by SRK activation, and the cellular mechanism by which incompatible pollen is rejected. Some progress has already been made in this area with the identification of ARC1 (a protein containing the arm-repeat protein-protein interaction domain), which shows a specific phosphorylation-dependent interaction with the kinase domain of SRK [24]. Antisense experiments demonstrated that ARC1 is required for the SI response in *Brassica* [25] but its function remains obscure. Two thioredoxin-h proteins, THL1 and THL2, that act to reduce disulfide bonds, also interact with the kinase domain of SRK in a phosphorylation-independent manner [26]; THL1 appears to inhibit the kinase activity of SRK in the absence of 'activating' components of the pollen coating (presumably SCR/SP11 of the same haplotype) [27]. A possible final target for cytosolic signals generated by the SRK-SCR/SP11 interaction is MOD, an aquaporin (water-channel protein) located in the plasma membrane of stigmatic papillae [28]. Selective control of water flow into pollen from the stigma has long been postulated to be critical in compatible and incompatible pollen-stigma interactions in *Brassica* [1,29], so differential regulation of water-channel proteins might be predicted to be a feature of pollen 'inhibition' during the *Brassica* SSI response. This and other questions relating to targets for SRK signaling will be the focus for the forthcoming new phase in *Brassica* SSI research.

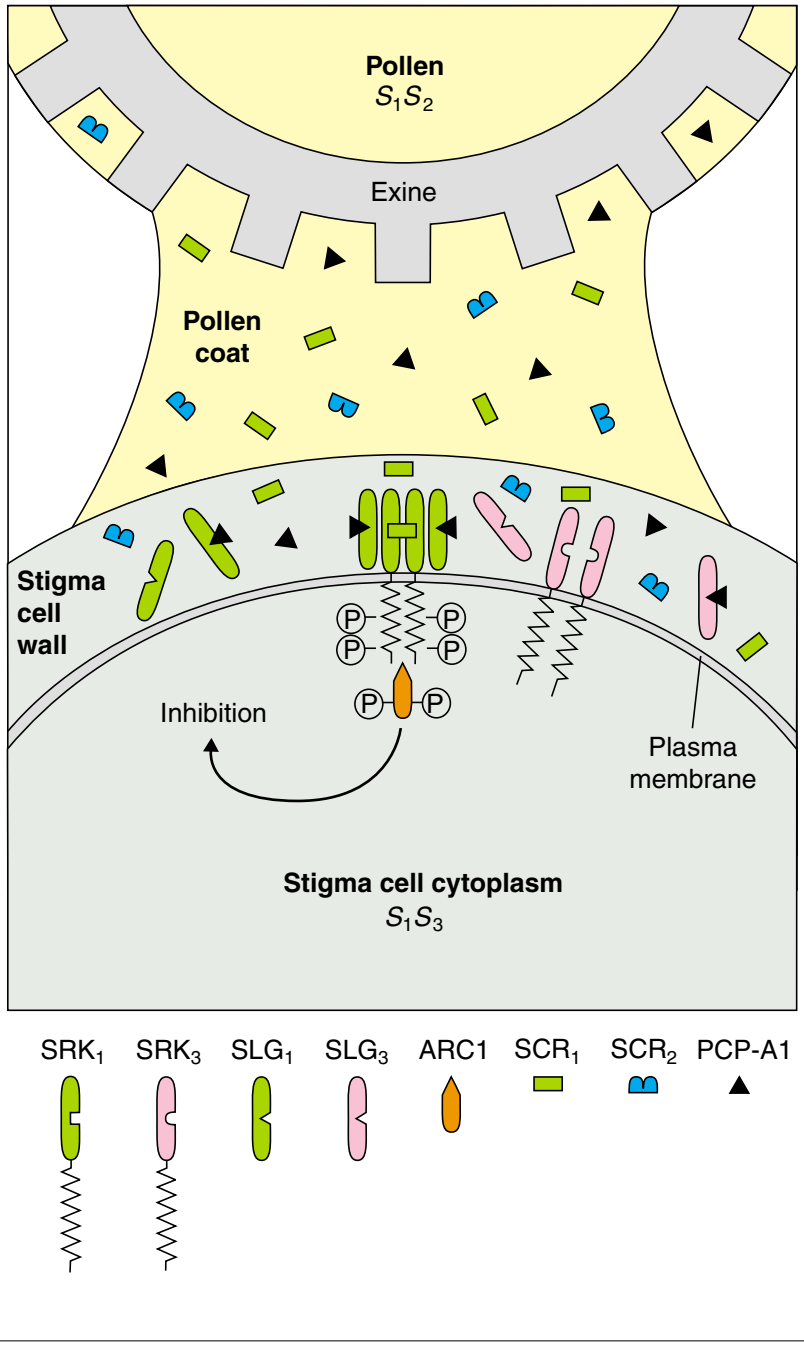

Figure 2
Model for the mechanism of SSI in *Brassica* species. Inhibition of incompatible pollen occurs at the stigma surface, usually before germination of the pollen, and appears to involve a deregulation of water flow to the pollen from the stigma during hydration of the pollen grain [29]. Here, pollen from an S_1S_2 individual is inhibited on an S_1S_3 stigma as a consequence of a haplotype-specific interaction between male (pollen) and female (stigma) products of the S_1 haplotype (see Figure 1b). The S proteins are color coded: S_1, green; S_2, blue; and S_3, pink. SCR_1, a pollen S protein, is recognized by, and binds to, the extracellular receptor domain of SRK_1, a stigma S protein, thereby inducing dimerization of SRK_1 and autophosphorylation (shown by P) on serine and threonine residues in the kinase domain (shown as a zig-zag tail). 'Activation' of the SRK protein then initiates an intracellular signaling cascade within the stigma that leads to localized rejection of the pollen. SLG acts as an accessory protein in the formation of the receptor complex (see text for further details). PCP-A1, a small cysteine-rich pollen-coat protein similar to SCR, but not genetically linked to S, binds nonspecifically to SLGs from all haplotypes and may function as an additional accessory protein alongside SLG during formation of the receptor complex. Signaling downstream of SRK has not yet been characterized, but essential for the SI response is ARC1, an arm-repeat protein, that binds to the kinase domain of SRK in a phosphorylation-dependent manner, but whose function is as yet unknown. Exine is the outer region of the pollen cell wall.

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