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Review

Self-incompatibility in Brassicaceae crops: lessons for interspecific incompatibility

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Most wild plants and some crops of the Brassicaceae express self-incompatibility, which is a mechanism that allows stigmas to recognize and discriminate against "self" pollen, thus preventing self-fertilization and inbreeding. Self-incompatibility in this family is controlled by a single *S* locus containing two multiallelic genes that encode the stigma-expressed *S*-locus receptor kinase and its pollen coat-localized ligand, the *S*-locus cysteine-rich protein. Physical interaction between receptor and ligand encoded in the same *S* locus activates the receptor and triggers a signaling cascade that results in inhibition of "self" pollen. Sequence information for many *S*-locus haplotypes in *Brassica* species has spurred studies of dominance relationships between *S* haplotypes and of *S*-locus structure, as well as the development of methods for *S* genotyping. Furthermore, molecular genetic studies have begun to identify genes that encode putative components of the self-incompatibility signaling pathway. In parallel, standard genetic analysis and QTL analysis of the poorly understood interspecific incompatibility phenomenon have been initiated to identify genes responsible for the inhibition of pollen from other species by the stigma. Herewith, we review recent studies of self-incompatibility and interspecific incompatibility, and we propose a model in which a universal pollen-inhibition pathway is shared by these two incompatibility systems.

Key Words: self-incompatibility, interspecific incompatibility, Brassicaceae, SLG, SRK, SCR/SP11, QTL analysis.

Introduction

Flowering plants have evolved a number of intraspecific and interspecific pre-fertilization pollination barriers that allow pistils to control which of the many pollen grains that arrive at the stigma can effect fertilization. The best-known intraspecific pollination barrier is self-incompatibility (De Nettancourt 2001), which is considered to have evolved as a mechanism for the avoidance of inbreeding depression and the maintenance of genetic variation in populations. For their part, interspecific incompatibility barriers prevent hybridization between different species and thus maintain the identity of each species.

The self-incompatibility response of the Brassicaceae has been well studied, especially in *Brassica rapa*, *B. oleracea*, and *B. napus*, which include major vegetable and oil crops, and also in the genus *Arabidopsis*, including the model plant *A. thaliana*. In this self-incompatibility system, incompatible pollen is manifested at the surface of stigma epidermal cells by the failure of pollen grains to germinate and produce pollen tubes that elongate into the epidermal cell wall (Fig. 1). Genetic studies carried out in the 1950s revealed that this trait is controlled by variants of a single locus, the *S* locus, (Bateman 1955). In the 1960s, immunochemical analysis of stigmas led to the identification of proteins encoded by genes at the *S* locus (Nasrallah and Wallace 1967a). And starting in the 1980s, molecular genetic analyses identified the *S*-locus genes that are responsible for the recognition of "self" pollen by the stigma. Many alleles of these genes have now been sequenced, and the sequence information has contributed to biological, physiological, biochemical, genomic, and genetic studies of self-incompatibility as well as to practical breeding utilizing this trait. Several review articles published recently have provided a general understanding of self-incompatibility in the Brassicaceae and other plant families (Iwano and Takayama 2012, Kitashiba and Nishio 2009, Rea and Nasrallah 2008, Tantikanjana and Nasrallah, 2012).

In the case of interspecific incompatibility in the Brassicaceae, cytological observations have been carried out (Hiscock and Dickinson 1993, Lewis and Crowe 1958), but the genetic basis of the phenomenon is not understood. However, because the manner in which incompatible pollen is inhibited by cells of the pistil is the same in intraspecific and interspecific pollination barriers, it has been suggested that the same or very similar signaling pathways underlie

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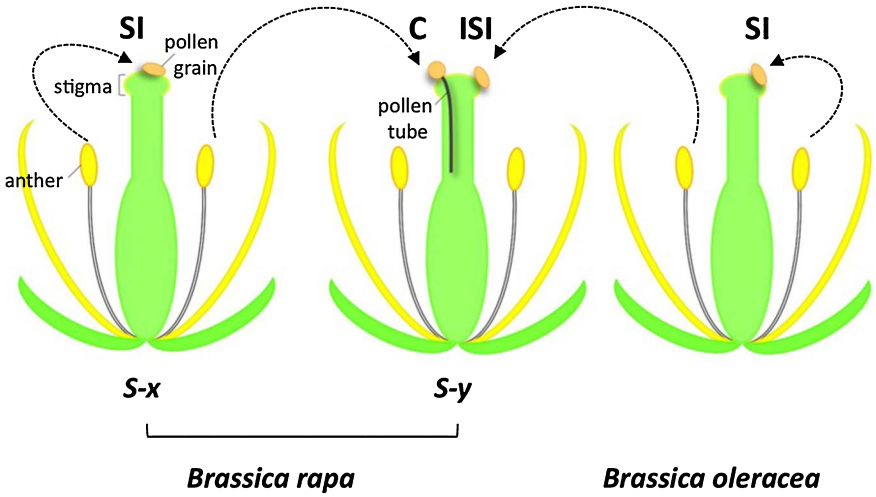

Fig. 1. Illustration of self-incompatibility and interspecific (interspecies) incompatibility in *Brassica* species. SI: self-incompatibility, C: compatibility, ISI: interspecific incompatibility.

pollen recognition in the two systems. Recently, the availability of genetic maps and genomic information for an increasing number of Brassicaceae species has spurred renewed efforts to understand the genetic basis of this trait. We herewith review recent studies of self-incompatibility and interspecific incompatibility, and we propose a model of a shared universal signaling pathway that might cause inhibition of conspecific and heterospecific pollen at the stigma surface.

Molecular genetics of self-incompatibility

In the Brassicaceae, the self-incompatibility phenotype of both stigma and pollen is determined sporophytically by the diploid *S*-locus genotype of the parent plant and dominance relationships between *S*-locus variants are observed in both stigma and pollen (Thompson and Taylor 1966). Pollen inhibition at the stigma surface occurs when the pollen and stigma express the same *S*-locus variant. Molecular genetic studies of the *Brassica S* locus identified three genes, two of which were shown by functional analyses to encode proteins responsible for specific recognition of "self" pollen by the stigma. The three genes are the *S*-locus receptor kinase (SRK), the *S*-locus cysteine rich protein/*S*-locus protein 11 (SCR/SP11), and the *S*-locus glycoprotein (SLG). Because the three genes are tightly linked to each other at the *S* locus and their alleles are inherited as one genetic unit, the term "*S* haplotype" has been coined to designate variant forms of the *S* locus (Nasrallah and Nasrallah 1993).

Alleles of *S*-locus genes and *S* haplotypes have commonly been represented with numerical subscripts, e.g. S_1, S_2, etc. However, according to standard nomenclature, alleles should be shown, not with subscripts, but with "+" or "–", e.g. *S+1* or *S-1* (Meinke and Koornneef 1997, Østergaard and King 2011). Hereafter, to designate *S* haplotypes and alleles of *S*-locus genes, we use "–" followed by the allele number and preceded by letters that abbreviate the species name: e.g., *S* haplotypes of *B. rapa* and *B. oleracea* are represented by *BrS-1* and *BoS-1*, respectively.

Identification of S-locus genes: SLG, SRK, and SCR/SP11

Of the three *S*-locus genes, the *S*-locus glycoprotein gene, *SLG*, was identified first. Its protein product was identified in stigma extracts by immunochemical analysis as *S* allele-specific antigens (Nasrallah and Wallace 1967b) and by electrophoretic analysis as having *S* allele-specific pI values (Nishio and Hinata 1977). In both cases, variant forms were shown to co-segregate with *S* alleles (Hinata and Nishio 1978, Nasrallah and Wallace 1967b). Subsequently, *SLG* cDNAs were cloned and sequenced (Nasrallah *et al.* 1985, 1987) and the amino-acid sequence of SLG was also determined by direct protein sequencing (Takayama *et al.* 1987). This initial sequence information enabled the determination of sequences for many additional *SLG* alleles in *Brassica* species and in *Raphanus sativus* (Chen and Nasrallah 1990, Kusaba *et al.* 1997, Sakamoto *et al.* 1998, Trick and Flavell 1989,). All *SLG* alleles are predicted to encode a primary translational product consisting of a hydrophobic signal peptide at the N-terminus for secretion to the outside of cells, several potential *N*-glycosylation sites, and twelve conserved cysteine residues. The mature SLG protein is a highly polymorphic glycoprotein having many residues that vary between alleles. Moreover, the *SLG* gene is expressed at high levels in stigma epidermal cells as demonstrated by *in situ* hybridization of *SLG* transcripts (Nasrallah *et al.* 1988) and analysis of *SLG* promoter activity using the GUS reporter (Sato *et al.* 1991), and the SLG protein accumulates in the wall of stigma epidermal cells as determined by immunocytochemical analysis (Kandasamy *et al.* 1989).

The next *S*-locus gene to be identified was *SRK*, which was isolated by screening a genomic library with an *SLG* probe and was found to contain a kinase-encoding sequence and to be linked to *SLG* (Stein *et al.* 1991). The primary SRK translational product consists of an N-terminal signal

sequence, an extracellular domain named "S" domain that is highly similar to SLG, followed by a transmembrane domain and a serine/threonine protein kinase domain toward the C-terminus. Like *SLG*, the *SRK* gene is predominantly expressed in stigma epidermal cells (Nasrallah *et al.* 1994). Also similar to SLG, the S domain of SRK is highly polymorphic, with some variants exhibiting more than 30% amino-acid sequence divergence. Comparison of nucleotide sequences and deduced amino-acid sequences demonstrated that the SRK S domain and the SLG encoded by the same *S* haplotype share more than 90% amino-acid sequence identity (Hatakeyama *et al.* 1998b; Sato *et al.* 2002, Stein *et al.* 1991, Watanabe *et al.* 1994).

The last *S*-locus gene to be identified was the *SCR/SP11* gene. In 1999, two groups, Schopfer *et al.* (1999) and Suzuki *et al.* (1999), surveyed the *S* locus by cloning and sequencing of the *S*-locus region in *B. rapa*, and identified a gene adjacent to *SLG* and *SRK* that exhibited anther-specific expression. Sequence analysis of this *SCR/SP11* gene predicted a protein having an N-terminal signal peptide, which when cleaved, would result in a mature cysteine-rich protein that is secreted, small in size (~50 amino acids), and basic in nature. Sequence comparisons showed that the mature SCR/SP11 protein is highly polymorphic, with less than 50% amino-acid similarity shared by variants within the same species (Okamoto *et al.* 2004, Sato *et al.* 2002, Schopfer *et al.* 1999, 2000, Watanabe *et al.* 2000). Only a few amino acids are conserved in most SCR/SP11 variants: eight cysteines, a glycine between the first and second cysteines, and an aromatic amino-acid residue between the third and fourth cysteines (Schopfer *et al.* 1999, 2000, Takayama *et al.* 2000, Watanabe *et al.* 2000). Furthermore, GUS reporter analysis of the *SCR/SP11* promoter (Schopfer *et al.* 2000) and *in situ* hybridization of *SCR/SP11* transcripts (Takayama *et al.* 2000, Kusaba *et al*. 2002) demonstrated that the gene was predominantly expressed in the anther tapetum, as expected for the sporophytic control of pollen self-incompatibility specificity in the Brassicaceae.

The S haplotypes of Brassica and Raphanus species

A large number of *S* haplotypes have been identified in *B. rapa* and *B. oleracea* and in the closely-related *Raphanus sativus* by pollination tests, electrophoretic analysis of stigma proteins, analysis of DNA polymorphism in *SLG*s or *SRK*s, and determination of *SLG*, *SRK*, and *SCR/SP11* sequences. In *B. oleracea*, among the 49 *S* haplotypes reported to date (Oikawa *et al.* 2011), sequences of the *SLG*, *SRK* or *SCR/SP11* alleles have been determined for all *S* haplotypes with the exception of four *S* haplotypes, *S-10*, *S-56*, *S-66* and *S-67*. In *B. rapa*, the sequences of the three genes have been determined for 44 *S* haplotypes (Oikawa *et al.* 2011). On the basis of the extent of sequence similarity shared by *SLG* and *SRK* alleles, *S* haplotypes have been grouped into two major classes, designated class-I and class-II *S* haplotypes (Nasrallah *et al.* 1991). Because of the availability of these sequences, *S* haplotypes have been standardized in *B. oleracea* and *B. rapa*, and collections of *S* haplotypes have been established in the two species. However, it is likely that additional *S* haplotypes exist which have not been characterized. Nou *et al.* (1993) used calculations based on the number of *S* haplotypes in wild populations in Japan and Turkey to estimate that more than 100 *S* haplotypes exist in *B. rapa*. Ockendon (2000) speculated that the total number of *S* haplotypes in *B. oleracea* is approximately 50 based on his long-time study of *S*-haplotype collections.

In contrast to the situation in *Brassica*, some confusion exists in the numbering of *S* haplotypes in *R. sativus*. Sakamoto *et al.* (1998) and Okamoto *et al.* (2004) reported on 20 *R. sativus S* haplotypes (*S-1* to *S-19* and *S-21*) along with the sequences of their *SLG*, *SRK*, and/or SCR/*SP11* alleles. Lim *et al.* (2002) also reported the sequences of these genes for 10 *S* haplotypes that they named *S-1* to *S-10*, but these *S* haplotype numbers do not refer to the same numbers used by Sakamoto *et al.* (1998) and Okamoto *et al.* (2004). Additionally, Niikura and Matsuura (1999) used pollination assays and PCR-RFLP analyses to identify 37 *S* haplotypes from Japanese, Asian, and European domesticated and commercial cultivars, and numbered them *S-201* to *S-237*. Since the latter *S* haplotypes are not associated with sequence information for the *SLG*, *SRK* and *SCR/SP11* alleles, it is not known if they are identical to any of the *S* haplotypes identified in the studies described above. Exchanges of plant materials between researchers and breeders and the establishment of a unified nomenclature of *S* haplotypes are necessary to avoid confusion regarding the identity of *S* haplotypes in *R. sativus*.

Owing to the extensive *S* haplotype collections in *Brassica* species, close relationships between SLGs and the S-domains of SRKs have been unveiled. The high degree of identity shared by the two sequences suggests the occurrence of gene conversion between *SLG* and the *S* domain of *SRK* in the same *S* haplotype. This conclusion is supported by analysis of the progeny of a *B. rapa* commercial cultivar showing self-compatibility, in which part of the *S* domain of *SRK* was replaced with the corresponding part of *SLG* from the same *S* haplotype (Fujimoto *et al.* 2006c). The occurrence of such gene conversion events has important implications. If a mutation causes an amino-acid substitution within the SRK S domain, which is the domain that perceives SCR (see below), the recognition specificity of SRK will be changed, causing a switch to self-compatibility. The resulting self-compatible plant would suffer inbreeding depression produced by repeated self-pollinations and is expected to become extinct. Therefore, to maintain recognition specificity, the mutation in SRK must be amended. Gene conversion between *SRK* and *SLG* is thought to provide a mechanism for the amendment of the mutated segment of the *SRK S*-domain by replacement with the corresponding non-mutated segment from *SLG* (Sato *et al.* 2002).

Comparison of nucleotide and amino-acid sequences of the *SLG*, *SRK*, and *SCR/SP11* alleles also contributed to the

identification of interspecific pairs of *S* haplotypes, i.e. pairs of *S* haplotypes from different species, whose *SLG*, *SRK*, and *SCR/SP11* sequences are more similar to each other than to those derived from other *S* haplotypes within the same species (Kimura *et al.* 2002, Kusaba *et al.* 1997). So far, eighteen interspecific pairs were found in comparisons of *S* haplotypes from *B. rapa* and *B. oleracea* (Kimura *et al.* 2002, Kusaba *et al.* 1997, Kusaba and Nishio 1999, Oikawa *et al.* 2011, Sato *et al.* 2003) and one intergeneric pair was identified by comparing *B. rapa* and *R. sativus* sequences (Okamoto *et al.* 2004, Sato *et al.* 2004). The use of several methods, including interspecific hybridization between *B. rapa* and *B. oleracea* (Kimura *et al.* 2002), transformation of *B. rapa* with *B. oleracea* or *R. sativus SCR/SP11* alleles, and bioassays of SCR/SP11 proteins synthesized in bacteria (Sato *et al.* 2003, 2004, 2006) have revealed that the interspecific pairs of *S* haplotypes retain the same recognition specificities. These reports suggest that each interspecific and intergeneric pair of *S* haplotypes originated from a common ancestral *S* haplotype, and that the diversification of *S* haplotypes occurred before speciation. Further insight into the diversification of *S* haplotypes will be obtained by analysis of *S*-haplotype collections in self-incompatible species of other genera of the Brassicaceae.

As the number of characterized *S* haplotypes increases, the development of methods for the identification of *S* haplotypes becomes increasingly important. Although classical methods, such as pollination tests and isozyme analysis, allow discrimination between *S* haplotypes, a method for analysis of DNA polymorphisms by PCR-RFLP, was developed based on polymorphisms in *SLG* and *SRK* alleles (Brace *et al.* 1993, Nishio *et al.* 1994). Because this method is simple and reliable, it is used for F_1 hybrid breeding and seed purity tests as well as for basic research on self-incompatibility. However, the PCR-RFLP method cannot easily identify the *S* genotypes of heterozygous plants because of complicated band patterns and it is not applicable to the few *S* haplotypes that lack *SLG* (Sato *et al.* 2002). To replace the PCR-RFLP method, Fujimoto and Nishio (2003) developed a dot-blot hybridization method that utilizes the highly variable sequences of *SCR/SP11* alleles. More recently, this method was improved to be more reliable and simpler (Takuno *et al.* 2010). It now enables allele-specific detection of *SCR/SP11* with high signal intensities and it can be used to identify most known *S* haplotypes of *B. rapa* (40 *S* haplotypes) and 33 *S* haplotypes of *B. oleracea* (Oikawa *et al.* 2011). Owing to these efforts, a classification system for *S* haplotypes and associated *S* tester lines is now available for *B. rapa* and *B. oleracea* (Oikawa *et al.* 2011).

Genomic structure of the Brassica S locus

Analysis of genomic organization in a small number of *S* haplotypes has shown that the physical size of the *S*-locus core region, i.e. the region containing the *SLG*, *SRK*, and *SCR/SP11* genes, tends to be shorter and less variable in *B. rapa* than in *B. oleracea* (Boyes and Nasrallah 1993, Suzuki *et al.* 2000a), due at least in part to the insertion of several retrotransposon-like sequences in *B. oleracea S* haplotypes (Fujimoto *et al.* 2006a, Kimura *et al.* 2002). However, further comparative genomic studies of a much larger number of *S* haplotypes is required to determine if these differences reflect true differences between species or simply variation among different *S* haplotypes within a species. In any case, sequence analysis of the *S*-locus region identified several genes of unknown function in regions flanking the *S*-core region, two of which, the *SP6* (*S*-locus protein 6) and the *SLL2* (*S*-locus-linked 2) genes, delimit the ends of the *S* haplotype.

Comparative analysis of class-I *S* haplotypes in *B. rapa*, *B. oleracea*, and *B. napus* (Boyes *et al.* 1997, Cui *et al.* 1999, Shiba *et al.* 2003) have shown that the genomic regions outside the *S*-locus core exhibit a high degree of synteny and sequence similarity between different *S* haplotypes, whereas the *S*-locus core region is highly polymorphic and enriched in *S*-haplotype-specific intergenic sequences. In both *B. rapa* and *B. oleracea*, the organization of the *S* locus differs substantially between class-I and class-II *S* haplotypes. In the class-I *BrS-8*, *BrS-9*, *BrS-46*, *BrS-47* and *BrS-54* haplotypes, the order of genes is *SLG–SCR/SP11–SRK* (Fujimoto *et al.* 2006a, 2006b, Suzuki *et al.* 1999, Takuno *et al.* 2007). In contrast, in the class-II *BrS-60* haplotype, the order of *SRK* and *SCR/SP11* was the reverse of that in class-I *S* haplotypes (Fukai *et al.* 2003). Furthermore, the direction of transcription of the *SRK*, *SCR/SP11*, and *SLG* genes in four *B. rapa* class-II *S* haplotypes is completely conserved, although the region between *SRK* and *SCR/SP11* is highly diverse (Kakizaki 2006).

The diversity observed in the structure and sequence of different *S* haplotypes within the same species is considered to contribute to the suppression of recombination in the *S*-locus region (Boyes *et al.* 1997). Such suppression of recombination is thought to be required for maintaining the linkage of matched alleles of the *S*-locus recognition genes and consequently for persistence of the self-incompatibility trait, because recombination events that disrupt the recognition gene complex are expected to produce non-functional recombinant *S* haplotypes. Plants harboring these non-functional *S* haplotypes are expected to exhibit increased homozygosity for deleterious mutations and eventual elimination of the recombinant *S* haplotype due to inbreeding depression (Boyes *et al.* 1997, Casselman *et al.* 2000).

However, a genetic analysis of the frequency of recombination across the *B. rapa S* locus did not show a reduced rate of crossovers within the *S*-locus region relative to flanking regions or other regions of the genome (Casselman *et al.* 2000). Moreover, although no recombination events have apparently involved *SCR/SP11* and the *S*-domain of *SRK*, the kinase domain of *SRK* and other *S* locus-linked genes not responsible for self-recognition have experienced recombination between *S* haplotypes (Takuno *et al.* 2007). Further studies are required to determine if recombination is actually suppressed in the *S*-locus region and if estimates of

recombination frequencies will differ depending on which *S* haplotypes or combinations of *S* haplotypes are analyzed.

S haplotypes in other self-incompatible species of the Brassicaceae: Arabidopsis species

Subsequent to the identification of *S*-locus genes in *Brassica*, orthologs of these genes were sought in other self-incompatible species of the Brassicaceae, especially *Arabidopsis lyrata*, and to a lesser extent *A. halleri*, *Capsella grandiflora*, and *Leavenworthia* (Chantha *et al.* 2013). The *S* locus and its genes have been particularly well characterized in the genus *Arabidopsis*, which is considered to be closer to ancestral species of the Brassicaceae than *Brassica*. To date, many complete or partial sequences of *SRK* and *SCR* alleles have been obtained from *A. lyrata* and *A. halleri* (Bechsgaard *et al.* 2006, Boggs *et al.* 2009a, Kusaba *et al.* 2001, Prigoda *et al.* 2005, Schierup *et al.* 2001, Tsuchimatsu *et al.* 2010). In addition, the basic features of the *Arabidopsis S* locus were described, first by structural and transcriptional analysis of two *A. lyrata S* haplotypes (Kusaba *et al.* 2001) and subsequently by sequence analysis of additional *A. lyrata S* haplotypes (Goubet *et al.* 2012, Guo *et al.* 2011).

These studies showed that, similar to *Brassica S* haplotypes, *A. lyrata S* haplotypes differ in the order and orientation of the *S*-locus recognition genes, as well as in the physical distances between these genes owing to the insertion of variable numbers and types of transposable elements (Goubet *et al.* 2012, Guo *et al.* 2011, Kusaba *et al*. 2001). However, the *S* locus of *Arabidopsis* species, as well as that of *Capsella* species (Nasrallah *et al.* 2007), differs from the *Brassica* and *Raphanus S* locus in two major features: it lacks an *SLG* gene and it is not flanked by homologues of the *SP6* and *SLL2* genes as in *Brassica*, but is rather delineated by the *ARK3* gene on one end and the U-box gene *PUB8* on the other end (Conner *et al.* 1998, Goubet *et al.* 2012, Guo *et al.* 2011, Kusaba *et al.* 2001). Thus, the *S* locus occupies a region of the *Arabidopsis* and *Capsella* genomes that is not syntenic with the *S*-locus region in *Brassica*, suggesting that a translocation of the locus occurred in *Brassica*, likely as a consequence of the extensive genomic rearrangements that are known to have occurred in this genus (Kusaba *et al.* 2001).

Functional analysis of the self-incompatibility response

S-locus genes and their role in self-recognition

The expression of the *SLG* and *SRK* genes in stigma epidermal cells and of the *SCR/SP11* gene in the tapetum is consistent with a role for the products of these genes as determinants of self-incompatibility specificity in stigma and pollen, respectively. Proof that genes actually fulfill these functions was obtained by transformation experiments. Thus, the *SCR/SP11* gene was shown to encode the pollen determinant of self-incompatibility specificity by Schopfer *et al.* (1999) and subsequently by Shiba *et al.* (2001): in both studies, pollen grains from plants transformed with an allele of this gene was shown to be specifically inhibited on stigmas expressing the corresponding SRK variant.

For SLG and SRK, determining a role in self-incompatibility was less straightforward. The self-incompatibility response is developmentally regulated as a function of stigma development, such that immature stigmas are self-compatible and the ability of the stigma to inhibit "self" pollen is first observed one day prior to anthesis. Both *SLG* and *SRK* transcripts are developmentally regulated, attaining maximal levels in the stigmas of flower buds just before anthesis, coincident with the onset of the self-incompatibility response (Delorme *et al.* 1995, Stein *et al.* 1991, Watanabe *et al.* 1994). This expression pattern, together with the high degree of polymorphism exhibited by both SLG and SRK, raised a question about which gene functioned as the stigma determinant of specificity in the self-incompatibility response. Initial attempts to modify self-incompatibility specificity by transformation with *SRK* genes were not successful due to transgene-mediated silencing, which caused suppression of the *SRK* transgene as well as the endogenous *SLG* gene (Conner *et al.* 1997). Subsequently, Takasaki *et al.* (2000) found that *B. rapa* plants that were transformed with *SRK-9* (equivalent to *SRK-28*) exhibited rejection of pollen grains from an *S-9* homozygote, while transgenic plants harboring the *SLG-9* transgene did not. In support of these results, several *Brassica* plants either having defects in SLG-coding sequences, e.g., *BoS-18* and *BoS-60*, or lacking an *SLG* gene, e.g., *BrS-32* and *BrS-36*, were found to exhibit a strong self-incompatibility response (Sato *et al.* 2002, Suzuki *et al.* 2000b). Furthermore, as noted earlier, the *S* locus of *Arabidopsis* and *Capsella* species lacks an *SLG* gene. These observations indicate that *SRK* is the sole female determinant of *S*-haplotype specificity in the self-incompatible response.

What then is the role of SLG? Transgenic *B. rapa* plants harboring both *SRK-9* and *SLG-9* transgenes exhibited a significantly enhanced self-incompatibility response compared with transgenic plants harboring the *SRK-9* transgene alone (Takasaki *et al.* 2000). However, no such enhancement of self-incompatibility was found in another transgenic experiment that used *SRK-910* and *SLG-910* in self-incompatible *Brassica napus* (Silva *et al.* 2001). Taken together, these observations indicate that although SLG is not necessary for the self-incompatibility response, it may enhance the SRK-mediated self-incompatibility response in some *S* haplotypes such as *BrS-9*, probably because of the high sequence similarity (98%) shared by SLG and the S domain of SRK in this haplotype. A possible molecular explanation for this enhancing effect of SLG was provided by Dixit *et al.* (2000). Two mutant self-compatible *B. oleracea* strains that expressed low levels of SLG were found to accumulate normal levels of *SRK* mRNA, yet failed to produce SRK protein. In addition, the *B. oleracea* BoSRK-6 variant was found to form aberrant high molecular mass

aggregates when expressed alone in tobacco leaves but not when co-expressed with BoSLG-6. These results suggest that for at least some SRK variants, SLG is important for the stabilization and proper processing of SRK and its accumulation to physiologically relevant levels in stigmas.

The fact that *SRK* and *SCR* are the sole determinants of self-incompatibility specificity was dramatically demonstrated by transfer of the self-incompatibility trait to *A. thaliana*, the self-compatible close relative of *A. lyrata* and *A. halleri. A. thaliana* harbors non-functional *S* haplotypes, designated pseudo-*S* (*ΨS*) haplotypes that contain non-functional alleles of SRK (*ΨSRK*) and/or SCR (*ΨSCR*) that either encode truncated proteins or are highly decayed and exhibit deletions or rearrangements (Boggs *et al.* 2009b, Dwyer *et al.* 2013, Kusaba *et al.* 2001, Sherman-Broyles *et al.* 2007, Shimizu *et al.* 2008, Tang *et al.* 2007).

Transformation with functional *SRK* and *SCR* transgenes isolated from *A. lyrata* or *C. grandiflora* was sufficient to confer a self-incompatibility phenotype in several accessions of *A. thaliana* (Boggs *et al.* 2009b, Liu *et al.* 2007, Nasrallah *et al.* 2002, 2004). Furthermore, an *A. thaliana* accession harboring a functional *SRK* allele and a non-functional *SCR* allele exhibited self-incompatibility when transformed with the corresponding functional *SCR* allele from *A. halleri* (Tsuchimatsu *et al.* 2010). These transgenic complementation experiments confirm that *SRK* and *SCR* alone determine self-incompatibility specificity. Moreover, because the self-incompatibility response exhibited by *A. thaliana SRK* and *SCR* transformants was as intense as that observed in naturally self-incompatible plants, it may be concluded that the SRK-mediated signal transduction pathway is active even in this self-compatible species. As described later, the availability of transgenic self-incompatible *A. thaliana* plants has allowed researchers to exploit the extensive genetic resources and ease of transformation of the *A. thaliana* model species for analysis of the recognition and response phases of self-incompatibility.

The basis of specificity in the self-incompatibility response: allele-specific interactions between SRK and SCR

Several studies have demonstrated that SCR/SP11 is the ligand for SRK. Takayama *et al.* (2001) and Kachroo *et al.* (2001) reported that SRK and SCR/SP11 proteins interacted only if they were derived from the same *S* haplotype. SRK tends to form oligomers, especially homo-oligomers, in unpollinated stigmas, i.e. in the absence of its SCR/SP11 ligand (Giranton *et al.* 2000, Naithani *et al.* 2007, Shimosato *et al.* 2007). Because SRK dimerization is critical for high-affinity binding of SCR/SP11 (Shimosato *et al.* 2007), the constitutive homo-oligomerization of SRK might serve to prime SRK for rapid activation upon ligand binding. In a search for amino-acid residues or domains that determine SRK specificity, four hypervariable (HV) regions, HV1, HV2, HV3 and the C-terminal variable region (CVR) were identified (Kusaba and Nishio 1999, Nishio and Kusaba 2000, Sato *et al.* 2002). Because many of these polymorphic residues have a high probability for being under positive selection (Sainudiin *et al.* 2005), they are surmised to be important for specificity. However, transgenic experiments in *A. thaliana* involving domain swapping between two pairs of *A. lyrata* SRK alleles and *in vitro* mutagenesis determined that only a very small subset of these polymorphic amino-acid residues located in two clusters within the HV1 and HV2 regions were necessary for specific recognition of self SCR/SP11 ligand and activation of the self-incompatibility response (Boggs *et al.* 2009c). Whether these results apply to all SRKs is not known and a general rule for the key amino acids responsible for self-recognition has yet to be clarified.

Two approaches were used in an attempt to find a key motif for self-recognition specificity in the SCR/SP11 protein. One approach involved comparative analysis of *SCR/SP11* sequences from interspecific pairs of *S* haplotypes (Kusaba *et al.* 1997, Kimura *et al.* 2002). Based on this analysis, six regions (Regions I to VI), each delimited by conserved cysteine residues, were assigned to SCR/SP11, and domain-swapping experiments revealed that Regions III and V are necessary for recognition of "self" SRK (Sato *et al.* 2004). A second approach to define the residues responsible for recognition specificity in SCR was implemented by Chookajorn *et al.* (2004). In this study, the *B. oleracea* SCR-6 and SCR-13 alleles were analyzed by domain swapping of regions between the conserved cysteines and by site-directed mutagenesis to generate chimeras and mutant forms of SCR/SP11. These engineered variants were tested by ELISA and pull-down assays for binding to the extracellular S domains of SRK-6 and SRK-13 and by pollination bioassays for their ability to elicit self-incompatibility on the stigmas of *S-6* and *S-13* homozygotes. Surprisingly, this study showed that SCR-13 specificity was determined by only four contiguous amino-acid residues in the region between the fifth and sixth conserved cysteines, which corresponds to Region V defined by Sato *et al.* (2004). However, the corresponding region in SCR-6 did not effect a change in specificity when inserted into the SCR-13 backbone. Thus, different regions or combinations of regions can determine specificity in different SCR/SP11s. Consequently, it has not been possible to infer general rules for defining residues that determine SCR/SP11 specificity.

Dominance relationships between *S* haplotypes in *Brassica* and *A. lyrata*

In their analysis of genetic dominance relationships between *B. oleracea* and *B. rapa S*-locus variants, Thompson and Taylor (1966) and Hatakeyama *et al.* (1998a) found that class-I *S* haplotypes are generally dominant to class-II *S* haplotypes in pollen. Molecular analysis in *Brassica* and in *A. lyrata* demonstrated that plants heterozygous for a dominant and a recessive *SCR/SP11* allele failed to accumulate transcripts derived from the recessive *SCR/SP11* allele in the anther tapetum, where *SCR/SP11* transcripts are normally

expressed (Kusaba *et al.* 2002, Shiba *et al.* 2002). This result indicates that transcription of a recessive *SCR/SP11* allele is suppressed in the presence of a dominant *SCR/SP11* allele. Interestingly, Fujimoto *et al.* (2006b) observed that class-I *S* haplotypes in which the *SCR/SP11* is not transcribed due to defects in promoter activity also caused suppression of recessive class-II *SCR/SP11* alleles, suggesting that expression of dominant *SCR/SP11* alleles is not necessary for the suppression of recessive *SCR/SP11* alleles. Subsequently, Shiba *et al.* (2006) demonstrated that suppression of the transcription of a recessive class-II *SCR/SP11* in heterozyogtes occurred epigenetically by *de novo* methylation of 5′ promoter sequences in tapetum cells. Further analysis of this phenomenon (Tarutani *et al.* 2010) identified inverted genomic sequences similar to the sequence of class-II *SCR/SP11* promoters in regions flanking the dominant *SLG* alleles, which produced an anther-specific trans-acting small non-coding RNA (small RNA). Furthermore, a transgene of this small RNA induced methylation of the promoter of recessive class-II *SCR/SP11* alleles. Taken together, these results indicate that the small RNA is a key sequence for suppression of the class-II *SCR/SP11* transcription.

However, this mode of epigenetic control may not be generally applicable to all cases of *S* haplotype dominance in pollen, as suggested by analysis of dominant-recessive interactions among *B. rapa* class-II *S* haplotypes. Four *B. rapa* class-II *S* haplotypes (*S-44*, *S-60*, *S-40* and *S-29*) have been reported, which constitute a linear dominance series, *BrS-44* > *BrS-60* > *BrS-40* > *BrS-29*, in which *BrS-44* is the most dominant and *BrS-29* is the most recessive (Kakizaki *et al.* 2003). Plants heterozygous for combinations of these class-II *S* haplotypes exhibited *de novo* promoter methylation and suppression of the transcription of recessive *SCR/SP11* alleles in anthers (Shiba *et al.* 2006). However, trans-acting non-coding RNA sequences having homology with the promoters of the recessive *SCR/SP11* alleles were not observed in the dominant *BrS-44* haplotype, and the non-coding RNA sequences in other *S* haplotypes were poorly transcribed (Tarutani *et al.* 2010). This observation suggests that the molecular mechanism that causes promoter methylation and suppression of transcription of class-II *SCR/SP11* alleles is not based on the activity of an *S*-locus linked small RNA species. Further sequence analysis of small RNAs and unidentified sequences in the recessive class-II *SCR/SP11* promoters will no doubt be informative in this regard.

As for the dominance relationships of *S* haplotypes in stigmas, genetic analyses have been conducted (Hatakeyama *et al.* 1998a, Thompson and Taylor 1966) but no molecular explanation has been put forth. Because recessiveness of *S* haplotypes in the stigma is not associated with lower *SRK* expression levels (Hatakeyama *et al.* 2001), it is possible that dominance relationships are determined, not by differences in relative expression levels of *SRK*, but by features of the SRK protein itself. In particular, the S domain rather than the kinase domain may dictate dominance relationships, as suggested by sequence analysis of SRKs derived from *S* haplotypes that exhibit different allelic relationships. For example, sequence analysis of *BrSRK-54*, which is co-dominant with *BrSRK-8* and recessive to *BrSRK-46*, has shown that the SRK kinase domain of these three variants are highly similar (98.3 to 100 % at amino acid level), while their S domains exhibit a much higher degree of sequence divergence (77.8 to 85.3 % at amino acid level) (Takuno *et al.* 2007 and our unpublished data). In support of this conclusion, a yeast two-hybrid interaction analysis of SRK S domains showed a preference for homodimerization (i.e. interaction with an identical S domain derived from the same SRK variant) over heterodimerization (i.e. interaction with an S domain derived from another SRK variant) (Naithani *et al.* 2007). This result suggested the hypothesis that SRK dominance relationships result from differences in the propensity of some pairs of SRKs to form heterodimers that might have reduced affinity for SCRs (Naithani *et al.* 2007). Further genetic and biochemical studies are required to test this hypothesis and to explain the puzzling dominance relationships of *SRK* alleles.

Self-compatibility of amphidiploid species in *Brassica* and *Arabidopsis*

The functionally diploid *B. rapa*, *B. nigra*, and *B. oleracea*, which have the A, B, and C genomes, respectively (U 1935), exhibit self-incompatibility. In contrast, *B. napus*, *B. juncea*, and *B. carinata*, which are amphidiploid species that contain the A and C genomes, the A and B genomes, and the B and C genomes, respectively, exhibit self-compatibility. Interestingly, artificially synthesized *Brassica* amphidiploid plants are self-incompatible (Hinata and Nishio 1980). To explain this discrepancy, 45 lines of *B. napus* were analyzed by cloning of *SLG*, *SRK*, and *SCR/SP11* genes. Seven *S* haplotypes were identified, among which *BnS-1* to *BnS-5* are class-I haplotypes while *BnS-6* and *BnS-7* are class-II *S* haplotypes (Okamoto *et al.* 2007). Four *B. napus* genotypes were combinations of a dominant class-I *S* haplotype and a recessive class-II haplotype (Okamoto *et al.* 2007, Tonosaki and Nishio 2010). Further analysis revealed that *S*-haplotype mutations were responsible for self-compatibility in three of these genotypes. In two *B. napus* genotypes, dominant *SRK* alleles had frame-shift mutations in their coding region that knocked out *SRK* function (Okamoto *et al.* 2007). By contrast, *B. napus* cv. 'Westar', which has the pollen-dominant *BnS-1* haplotype in its A genome and the pollen-recessive *BnS-6* haplotype in its C genome, contains functional *SRK* genes but has suffered loss of *SCR/SP11* function for two reasons: firstly, the dominant *BnS-1 SCR/SP11* allele is not expressed due to an insertion mutation in its promoter region and secondly, the recessive *BnS-6 SCR/SP11* allele is suppressed by the dominant *BnS-1 SCR/SP11* allele (Okamoto *et al.* 2007). This case of suppression is similar to the suppression of a recessive *SCR/SP11* allele by a dominant nonfunctional *SCR/SP11* in monogenomic

B. rapa described by Fujimoto *et al.* (2006b). In support of the conclusion that self-compatibility in 'Westar' is due to loss of *SCR/SP11* function, 'Westar' plants acquired the self-incompatibility trait when transformed with a functional *SCR/SP11* allele from the *BrS-47*, which is the likely *B. rapa S* haplotype progenitor of *BnS-1* (Tochigi *et al.* 2011). Taken together, these results demonstrate that a single mutation event in the *SRK* or *SCR/SP11* genes of a dominant *S* haplotype can cause self-compatibility in amphidiploid *Brassica* plants.

Self-compatible amphidiploids have also been described in other Brassicaceae. *Arabidopsis suecica*, which is an amphidiploid species derived by spontaneous hybridization between *A. thaliana*, a self-compatible species, and *A. arenosa*, a self-incompatible species, is self-compatible (Mummenhoff and Hurka 1995, O'Kane *et al.* 1996). In addition, artificial amphidiploids generated by crossing *A. thaliana* and *A. lyrata* also exhibited self-compatibility (Nasrallah *et al.* 2007) due to suppression of *A. lyrata*-derived *SRK* transcripts. Because backcrossing of the amphiploid to *A. lyrata* caused a reversion to self-incompatibility along with restoration of *SRK* expression, it is likely that an epigenetic change in the *A. lyrata SRK* gene or an *A. thaliana*-derived factor caused suppression of *SRK* gene expression (Nasrallah *et al.* 2007). Thus, based on the limited information available so far, it appears that the mechanism causing self-compatibility in amphidiploid *Arabidopsis* species is different from that observed in *Brassica* amphidiploids.

The self-incompatibility signaling pathway

While the stigma receptor kinase SRK, and its pollen ligand SCR/SP11 have been well characterized as determinants of self-recognition, the signaling pathway triggered by ligand binding and activation of the receptor and the mechanism of rapid self-pollen rejection are poorly understood. Nevertheless, molecular genetic analyses have implicated three proteins in self-incompatibility signaling: the M-Locus Protein Kinase (MLPK), the Arm repeat-Containing protein (ARC1), and the Exo70A1 component of the exocyst complex.

Classical genetic studies indicated that self-compatibility in *B. rapa* cv. 'Yellow Sarson', an Indian oil crop, is caused by two independent loci, the *S* locus, which contains non-functional recognition genes and the *M* locus (Hinata *et al.* 1983). Map-based cloning of the *M*-locus region identified a gene encoding a membrane-anchored cytoplasmic protein kinase, which was designated MLPK (Murase 2004). The *MLPK* gene produces two isoforms, both of which interact with SRK (Kakita *et al.* 2007). A single mutation causing a nonsynonymous substitution was found in the kinase domain of the 'Yellow Sarson' MLPK variant, which abolished autophospholylation activity and prevented localization of the protein to the cell membrane (Murase *et al.* 2004). Transient expression of a wild-type *MLPK* allele introduced by particle bombardment into the stigma epidermal cells of mutant *mlpk* homozygotes conferred a self-incompatibility phenotype in these cells. This result suggests that MLPK is important for the self-incompatibility response (Kakita *et al.* 2007). However, a stable transformation experiment that shows complementation of the self-compatibility trait by transformation of *mlpk* homozygotes with the wild-type *MLPK* gene has not been reported.

ARC1 was isolated in a yeast two-hybrid screen as a protein that interacts with and is phosphorylated by the SRK kinase domain (Gu *et al.* 1998). *ARC1* is expressed specifically in stigma tissues and antisense suppression of *ARC1* expression in a self-incompatible *B. napus* strain was reported to cause partial breakdown of self-incompatibility (Stone *et al.* 1999). ARC1 is an E3 ubiquitin ligase and is thus considered to ubiquitinate factors required for compatible pollination and cause their degradation via the proteasome protein degradation system (Stone *et al.* 2003). An *ARC1* ortholog was identified in a region of the *A. lyrata* genome that shares a high degree of synteny with the *Brassica ARC1* genomic region (Kitashiba *et al.* 2011), and transformation of *A. lyrata* plants with an *ARC1*-RNAi construct caused reduced transcription of the endogenous *ARC1* gene and partial loss of self-incompatibility (Indriolo *et al.* 2012). Additionally, *ARC1* sequences were found to be deleted in the genomes of some self-compatible Brassicaceae species, such as *Thellungiella parvula* and *Aeyhionema arabicum*. By providing a link between loss of ARC1 and self-compatibility, these results suggest that ARC1 functions in self-incompatibility signaling.

Exo70A1 was identified as an ARC1-interacting protein in a yeast two-hybrid screen (Samuel *et al.* 2009). Overexpression of *Exo70A1* in the stigma epidermal cells of self-incompatible *B. napus* reportedly caused partial breakdown of self-incompatibility. Furthermore, suppression of *Exo70A1* expression in self-compatible *B. napus* and *A. thaliana* using RNAi and T-DNA insertions, respectively, reportedly resulted in inhibition of pollen adhesion, hydration, and germination (Samuel *et al.* 2009). Because Exo70A1 is a component of the exocyst complex, which generally functions in polarized secretion in yeast and animals (Munson *et al.* 2006, Synek *et al.* 2006), the results suggested that factors required for compatible pollination are secreted to the outside of stigma epidermal cells through the exocyst complex. In self-incompatible plants, activation of SRK would cause activation of ARC1, which would ubiquitinate Exo70A1 and target it for degradation, thus preventing the secretion of factors required for proper hydration or germination of pollen grains.

Interestingly, very different results were obtained in an analysis of the role of the *A. thaliana* orthologs of MLPK, ARC1, and Exo70A1 using the transgenic *A. thaliana SRK-SCR* self-incompatible model (Kitashiba *et al.* 2011). *A. thaliana* contains a functional *MLPK* ortholog, *AtAPK1b*, which is located on chromosome 2 in a region that exhibits a high degree of synteny with the *MLPK* region of *B. rapa*. However, self-incompatibility was neither lost nor weakened

in *A. thaliana SRK-SCR* plants that were homozygous for an *AtAPK1b* allele inactivated by T-DNA insertion (Kitashiba *et al.* 2011), indicating that a functional *AtAPK1b* is not required for self-incompatibility in the stigmas of *A. thaliana SRK-SCR* plants. In the case of *ARC1*, comparative analysis of the *A. thaliana* and *A. lyrata* genomes identified only fragmented sequences interspersed with other genes in the *A. thaliana* Col-0 and C24 accessions (Kitashiba *et al.* 2011), and the same fragmented organization of *ARC1* sequences was also observed in all 96 *A. thaliana* accessions analyzed using PCR markers (Indriolo *et al.* 2012). In view of the fact that *A. thaliana* stigmas that express a functional *SRK* gene can exhibit an intense self-incompatibility response (Boggs *et al.* 2009a, Nasrallah 2002, 2004, Tsutimatsu *et al.* 2010), the results indicate that successful operation of the self-incompatibility signaling pathway in *A. thaliana* does not require a functional ARC1 protein. Nor does it require AtPUB17 (Rea *et al.* 2010), which is the member of the Plant U-Box family that exhibits the highest degree of sequence identity to *B. napus* ARC1 (Indriolo *et al.* 2012, Kitashiba *et al.* 2011).

Also contrary to the results obtained in *B. napus* (Samuel *et al.* 2009), overexpression of *AtExo70A1* in stigma tissues of self-incompatible *A. thaliana SRK-SCR* plants did not weaken the self-incompatibility response (Kitashiba *et al.* 2011). Thus, it appears that the *MLPK/ARC1/Exo70A1*-based model of self-incompatibility signaling that was proposed for *Brassica* does not apply to the self-incompatibility response of *A. thaliana SRK-SCR* plants.

How might the discrepancies between the results obtained in *Brassica* and *A. thaliana* be reconciled? A possibility is that the experiments aimed at suppression of *MLPK* and *ARC1* might not have been specific for the targeted genes and that *A. thaliana* genes related to the tested *MLPK* and *ARC1* homologues might have assumed the role proposed for *Brassica MLPK* and *ARC1* (Kitashiba *et al.* 2011). Another possibility is that self-incompatibility signaling is based, not on a single linear pathway, but on multiple signaling pathways (Tantikanjana *et al.* 2010), as reported for the plant defense response and signal transduction pathways in metazoans. These pathways might each contribute only partially to the overall self-incompatibility response, and different branches of the pathway might be utilized preferentially in different species of the Brassicaceae. Re-examination of the roles of the postulated signaling genes and their relatives in *Brassica* species and *A. thaliana* is required to distinguish between these possibilities.

Recently, mutagenesis of *SRK-SCR* transformants of the *A. thaliana* Col-0 accession revealed that a pathway involving small inhibitory RNAs controls self-incompatibility (Tantikanjana *et al.* 2009). Unlike *SRK-SCR* transformants of the C24, Cvi, Sha, Kas, and Hodja accessions, whose stigmas express an intense self-incompatibility response that persists throughout stigma development, Col-0[*SRK-SCR*] transformants express self-incompatibility in mature flower buds and very young flowers, but not in older stigmas. Because Col-0[*SRK-SCR*] transformants set seed, they were used for mutagenesis of the self-incompatibility trait using the standard approach of treating seed with a chemical mutagen (Tantikanjana *et al.* 2009). A screen for plants exhibiting a modified self-incompatibility response identified a mutation that enhanced the self-incompatibility phenotype of Col-0[*SRK-SCR*] transformants without affecting expression of the *SRKb* and *SCRb* transgenes (Tantikanjana *et al.* 2009). This mutation inactivated the RNA-dependent RNA polymerase RDR6, which functions in the production of trans-acing short interfering RNAs (ta-siRNAs). Interestingly, a mutation in *ARGONAUTE7* (*AGO7*), which functions downstream of *RDR6* and is specifically responsible for the production of ta-siRNA targeting Auxin Response Factors (ARFs), also caused enhanced self-incompatibility in Col-0[*SRK*-SCR] transformants (Tantikanjana *et al.* 2009). Furthermore, overexpression experiments showed that *ARF3*, a member of the ARF family, acts non-cell-autonomously from its site of expression in cells below the stigma to enhance the self-incompatibility response and simultaneously down-regulate auxin responses (Tantikanjana and Nasrallah 2012). These results suggest the involvement of auxin in the regulation of self-incompatibility. Further analysis of mutants, either those that occur in natural populations (Isokawa *et al.* 2010) or those induced by mutagenic treatments (Strickler *et al.* 2013, Tantikanjana *et al.* 2009) will no doubt identify new components of the SRK-mediated signaling pathway and clarify the mechanism of the self-incompatibility response.

Interspecific Incompatibility in the Brassicaceae

Genetic studies for identifying genes responsible for interspecific incompatibility in Brassica

In the interspecific incompatibility observed between the pollen and pistil of different species, the inhibition of incompatible pollen is cytologically identical to that observed in the self-incompatibility response. For example, the inhibition of both conspecific "self" pollen and heterospecific pollen occurs during pollen tube growth within the style in the Solanaceae and by arrest of pollen germination and tube penetration into the stigma epidermal cell wall in the Brassicaceae. Interestingly, interspecific incompatibility is often unilateral (Lewis and Crowe 1958), i.e. the pistils of self-incompatible species generally inhibit pollen from self-compatible species, while the reciprocal pollinations are not inhibited. Genetic studies of unilateral incompatibility in tomato, a member of the Solanaceae, have revealed that this trait is controlled in the style by quantitative trait loci (QTL) (Bernacchi and Tanksley 1997, Li and Chetelat 2010), one of which corresponds to the *S-RNase* gene, which is the stylar determinant of self-incompatibility in the Solanaceae (Bernacchi and Tanksley 1997). Furthermore, two genes encoding pollen factors involved in interspecific incompatibility were identified: one gene was linked to the *S* locus on chromosome 1, while the other was located on chromosome

6 (Chetelat and De Verna 1991) and found to be *Cullin 1*, which is highly similar to a pollen factor that functions in the self-incompatibility response in *Petunia* (Li and Chatelat 2010). These results identify substantial overlaps in the pathways that underlie pollen inhibition in self-incompatibility and interspecific incompatibility in the Solanaceae.

In the Brassicaceae, interspecific incompatibility has been observed in interspecific pollinations between *B. rapa* and *B. oleracea*. In addition, one case of unusual unilateral intraspecific incompatibility was reported in *B. rapa* (Takada *et al.* 2013), which will not be discussed further. In all cases of interspecific unilateral incompatibility that have been examined, only cytological and genetic analyses have been performed, and the molecular basis of pollen inhibition is not known. Nevertheless, some studies have even invoked the involvement of the *S* locus in unilateral interspecific incompatibility (Hiscock and Dickinson 1993).

Genetic analysis of *Brassica* interspecific incompatibility has provided some information on the stigma's ability to discriminate against heterospecific pollen, but as yet nothing is known about pollen factors that might be involved in this process. Udagawa *et al.* (2010) identified two strains of *B. rapa* that differed in the response of their stigmas towards heterospecific *B. oleracea* pollen: one strain exhibited a strong interspecific incompatibility response and its stigmas were highly inhibitory to *B. oleracea* pollen, while the other strain did not exhibit interspecific incompatibility and its stigmas allowed profuse pollen tube growth when pollinated with *B. oleracea* pollen. The interspecific incompatibility trait in stigmas was found to be dominant and controlled by quantitative trait loci (QTL). Five QTL explained over 75% of the phenotypic variance between the two strains, with one QTL on linkage group 2 explaining 32% of this variance. Contrary to the expectation that interspecific incompatibility might be based on the activity of ARC1 as proposed for self-incompatibility in *Brassica*, only a peak with non-significant LOD score was observed at the *ARC1* locus. Moreover, in another *B. rapa* population derived from a cross between the self-compatible 'Yellow Sarson' strain and a self-incompatible strain, the strength of interspecific incompatibility was independent of genotypic composition at the *M* locus and its *MLPK* gene (Udagawa *et al.* 2010). Thus, no evidence has been found so far for the sharing of components between the self-incompatibility and interspecific signaling pathways in *Brassica*. A firm conclusion on this issue must await the molecular cloning of genes underlying the interspecific incompatibility QTL as well as of genes that encode components of the self-incompatibility pathway.

Model of a shared signaling pathway for self-incompatibility and interspecific incompatibility

Despite the lack of supporting data, the parallels between pollen inhibition in self-incompatibility and interspecific incompatibility observed among species of the Brassicaceae are suggestive of the existence of a stigma-based response pathway that is shared, at least in part, by the two incompatibility systems. Clearly however, the recognition of "self" pollen and of heterospecific pollen must be mediated by distinct factors in stigma and pollen. Although the identity of these factors is not known, it may be assumed that, unlike SCR, which induces the self-incompatibility response, any factor in heterospecific pollen that might trigger an incompatibility response in the stigma must exhibit limited intraspecific polymorphism but extensive polymorphism between species and genera. This assumption is based on the fact that interspecific incompatibility analyzed in *B. rapa* is typically characterized by a uniform response to heterospecific pollen, irrespective of its origin. Thus, in the Udawaga *et al.* (2010) study, the pollen of all *B. oleracea* strains tested was inhibited on the stigmas of the *B. rapa* strain that expressed interspecific incompatibility but not on the stigmas of the strain that did not exhibit interspecific incompatibility. Moreover, *B. rapa* strains that express the interspecific incompatibility trait uniformly inhibited pollen derived from plants belonging to several species and genera of the Brassicaceae, such as *B. nigra*, *R. sativus*, *Eruca sativa* (our unpublished data). And all *B. rapa* strains analyzed in these studies were compatible with conspecific pollen derived from plants that express different *S* haplotypes (Udagawa *et al.* 2010).

Here we present a highly speculative model of a universal pollen-inhibition pathway that is shared by self-incompatibility and interspecific incompatibility. The model makes the following assumptions: (1) interspecific incompatibility involves recognition of a species-specific pollen factor by a stigma factor; (2) the stigma factor responsible for recognition of heterospecific pollen is a receptor protein kinase which is located in the plasma membrane of stigma epidermal cells; (3) the interaction between the stigma receptor and the species-specific pollen factor activates the same "universal" signaling pathway that causes inhibition of pollen in the self-incompatibility response, and (4) the factor found in conspecific pollen is a negative regulator of receptor activity. As shown in Fig. 2, in unpollinated stigmas, the receptor would be active and the universal pollen-inhibition pathway would be activated, i.e. it is in the 'On' state. This pathway will remain 'On' in the presence of heterospecific pollen because the receptor cannot recognize the pollen factor from other species. However, in pollinations with conspecific "nonself" pollen (i.e. pollen derived from plants of the same species that express *S* haplotypes that are different from those expressed in the stigma), the receptor kinase recognizes the conspecific pollen factor, whereby it is inactivated, causing the pollen-inhibition pathway to be turned to the 'Off' state and allowing pollen tube development.

Notably, this hypothetical scheme differs substantially from the accepted mode of SRK activation in the self-incompatibility response. Whereas SRK, which is inactive until bound by its cognate SCR, the receptor kinase

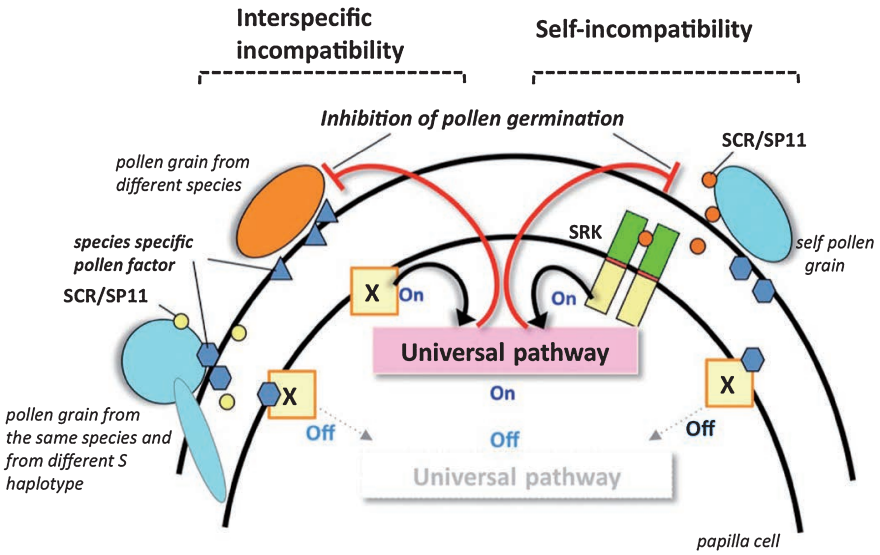

Fig. 2. Model of signaling thorough a hypothesized universal pathway in *Brassica* self-incompatibility and interspecific incompatibility. X is a hypothesized receptor kinase.

proposed for interspecific incompatibility is postulated to be constitutively active and to be inactivated by interaction with a species-specific pollen factor. This model of negative regulation of receptor activity overcomes some of the issues associated with a model based on receptor activation by a species-specific pollen factor, which would require the existence in the stigma of a large number of receptors, each of which would recognize, and become activated by, a pollen factor specific to a particular species. In any case, the proposed mode of receptor regulation resembles the regulation of the ethylene receptor ETR1, which is constitutively active in the absence of ethylene and switched off in the presence of ethylene (Yoo *et al.* 2009).

What might the identity of the postulated receptor be? The genomes of Brassicaceae and other plant species contain large numbers of genes encoding receptor kinases that are similar to SRK in sequence and structure (Shiu *et al.* 2004). For example, 38 such genes are found in the *A. thaliana* genome (Xing *et al.* 2013). It is possible that one of these SRK-like receptor kinases might function as a stigma receptor in interspecific incompatibility. It is hoped that continued molecular genetic analysis of *Brassica* and *Arabidopsis* species will elucidate the mechanisms that underlie pollen inhibition in interspecific incompatibility and self-incompatibility and clarify any overlaps that might exist between mechanisms of pollen inhibition in the two systems.

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