Review

Recent Progresses on Self-incompatibility Research in *Brassica* Species

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Many hermaphrodite plant species have evolved mechanisms to prevent self-fertilization. One such mechanism is self-incompatibility (SI), which is defined as the inability of a fertile hermaphrodite plant to produce zygotes after self-pollination. SI prevents self-fertilization by rejecting pollen from plants with the same genotype. The SI system in *Brassica* is controlled sporophytically by multiple alleles at a single locus, designated as *S*, and involves cell-cell communication between male and female. When the *S* phenotype of the pollen is the same as that of the stigma, pollen germination and/or pollen tube penetration are disturbed on the papilla cells. On the female side, two genes (*SLG* and *SRK*) located at the *S* locus, are involved in the SI reaction. *SLG* is a secreted glycoprotein expressed abundantly in the papilla cell, and *SRK* is a membrane-spanning receptor-like serine/threonine kinase whose extracellular domain is highly similar to *SLG*. Gain-of-function experiments have demonstrated that *SRK* solely determines *S* haplotype-specificity of the stigma, while *SLG* enhances the recognition reaction of SI. The sequence analysis of the *S* locus genomic region of *Brassica campestris* (syn. *rapa*) has led to the identification of an anther-specific gene, designated as *SP11*, which encodes a small cysteine-rich basic protein. Pollination bioassay and gain-of-function experiments have indicated that *SP11* is the male *S* determinant. When the sequence of *SP11* was aligned, six cysteine residues were found to be completely conserved among alleles. These conserved cysteine residues could be important for the tertiary structure of *SP11*. Recent biochemical analysis has suggested that *SP11* operates as a sole ligand to activate its cognate *SRK* specifically. Because the activity of the *S* allele is controlled sporophytically, dominance relationships influence the ultimate phenotype of both the stigma and pollen. Molecular analysis has demonstrated that the dominance relationships between *S* alleles in the stigma were determined by *SRK* itself, but not by the relative expression level. In contrast, in the pollen, the expression of *SP11* from the recessive *S* allele was specifically suppressed in the *S* heterozygote, suggesting that the dominance rela-

tionships in pollen were determined by the expression level of *SP11*.

Key Words: *Brassica* species, cell-cell recognition, dominance relationships, self-incompatibility, *S* haplotype, *S* locus.

Introduction

Flower is important for reproduction in angiosperms, and its shape is highly differentiated among the plant species. More than 70% of the species have hermaphrodite flowers for reproduction (de Nettancourt 2001). However, the structure of hermaphrodite flowers makes self-pollination highly possible. Self-pollination could lead to a high-risk situation in which the genetic diversity would decrease in the species. To prevent inbreeding and promote outcrossing, angiosperms have developed well-organized systems such as self-incompatibility (SI). SI is defined as the inability of a fertile hermaphrodite plant to produce zygotes after self-pollination (de Nettancourt 2001). In other words, SI is a system of discrimination between self-pollen and non-self-pollen on/in the pistil. The SI system in *Brassica* species is sporophytically controlled by a single polymorphic locus, designated as the *S* locus (Bateman 1955). When the pollen has the same *S* phenotype on the stigma, pollen germination and/or pollen tube penetration are disturbed at the stigma surface on the papilla cells (Fig. 1). SI has attracted considerable attention since this phenomenon was observed in detail by Darwin (1876, 1877). Furthermore, SI has been skillfully utilized in the breeding of hybrid varieties of economically important vegetables of Brassicaceae, namely cabbage, broccoli, cauliflower (*Brassica oleracea*), Chinese cabbage, turnip (*B. campestris* (syn. *rapa*)), radish (*Raphanus sativus*), and others. Thus, as an agricultural trait, SI is also considered to be very important.

In *Brassica* species, during the last two decades, molecular analysis of the *S* locus has been performed for identifying the male and female *S* determinants of SI, and as a result, both *S* determinants have been isolated and characterized. This review focuses on the recent studies dealing with the molecular mechanisms of SI in *Brassica* species. Many review articles published recently have also provided a general

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Fig. 1. Representative SI reaction in *B. campestris* (syn. *rapa*) L. Photographs were obtained by UV fluorescence microscopy. A, Cross-pollination of an S^{29} stigma with S^{60} pollen (compatibility); B, Self-pollination of an S^{60} stigma with S^{60} pollen (incompatibility). (Courtesy of T. Kakizaki (unpublished data)).

understanding of this field (Watanabe *et al.* 1999a, Watanabe and Hinata 1999, Watanabe *et al.* 2000b, Watanabe *et al.* 2001, Takayama and Isogai 2003, Isogai and Hinata 2002, Kachroo *et al.* 2002, Takayama and Sakagami 2002).

Identification and functional characterization of female S determinant

Identification of the *S*-specific antigen or the *S* locus-specific glycoprotein (later designated as *S* locus glycoprotein; SLG) in the stigma has enabled the analysis of this SI system by using molecular biological, molecular genetic, and biochemical techniques (Nasrallah and Wallace 1967, Nishio and Hinata 1977). SLGs encoded by respective *S* alleles were found to have different pI values (Nishio and Hinata 1977), and they co-segregated with *S* alleles without exception (Nou *et al.* 1993). They accumulated in the mature papilla cell wall, where the inhibition of self-pollen tube development occurs (Kandasamy *et al.* 1989, Kishi-Nishizawa *et al.* 1990). The SLGs consisted of a cleavable signal peptide, several *N*-glycosylation sites, three hypervariable regions, and twelve conserved cysteine residues towards the C-terminus (Takayama *et al.* 1987, Nasrallah *et al.* 1987). The function of SLG could not be predicted based on the sequence similarity to the other proteins.

The finding of *ZmPK1* in maize, which encodes a receptor-like protein kinase with an extracellular domain similar to SLG (Walker and Zhang 1990), led to the identification of the second *S*-linked gene, the *S* receptor kinase gene (*SRK*; Stein *et al.* 1991). The extracellular domain (designated as S domain, SD) of SRK showed a high similarity to SLG, and was connected via a single-pass transmembrane domain to a serine/threonine type protein kinase catalytic domain (Stein *et al.* 1991). The expression of *SRK* was specifically detected in stigma tissues, like the *SLG* gene (Watanabe *et al.* 1994). Based on the structural characteristics of SRK, the interaction between the S domain of SRK and the unidentified male *S* determinant is expected to trigger a phosphorylation cascade in the papilla cell that leads to the rejection of self-pollen.

Nucleotide sequence similarity between *SLG* and the region encoding the S domain of SRK derived from the same *S* allele was about 90%, and in some cases exceeded 98% (Stein *et al.* 1991, Watanabe *et al.* 1994, Hatakeyama *et al.* 1998c). The phylogenetic analysis of the *SLG* and *SRK* genes demonstrated that diversification of these genes predated the speciation in *Brassica* species (Hinata *et al.* 1995, Kusaba *et al.* 1997), and that intragenic recombination and point mutations have contributed to the generation of their sequence variation (Kusaba *et al.* 1997). Furthermore, gene conversion might occur frequently between the *SLG* and *SRK* genes (Watanabe *et al.* 1994, Suzuki *et al.* 1997a, Hatakeyama *et al.* 1998c).

Because the *S* locus consists of genes encoding both female and male *S* determinants within one segregational unit as described below, “*S* allele” is referred to as “*S* haplotype.” From the nucleotide sequence similarity of *SLGs/SRKs*, *S* haplotypes have been classified into two groups, class I and class II (Nasrallah *et al.* 1991). The sequence similarity among *SLGs/SRKs* within each class is about 80–90%, but that between classes is about 65%. In spite of the specific expression of both *SLG* and *SRK* genes in stigma tissues, the classification is correlated with dominant/recessive relationships on the pollen side (Hatakeyama *et al.* 1998a, 1998b): that is, class-II *S* haplotypes tend to be recessive to class-I *S* haplotypes on the pollen side.

Loss-of-function and gain-of-function experiments have been conducted to determine the role of *SLG* and *SRK* in the SI recognition reaction. In the case of the loss-of-function experiments, transgenic plants with antisense *SLG* gene and self-compatible mutants were analyzed. In transgenic *B. campestris* with antisense *SLG* driven by the *SLG* promoter, the level of transcripts of *SLG* and *SRK* decreased, and the transformants became self-compatible (Shiba *et al.* 1995, Shiba *et al.* 2000, Takasaki *et al.* 2001). In self-compatible mutants of *Brassica* species, the transcripts of *SLG* and/or *SRK* were down-regulated or undetectable (Nasrallah *et al.* 1992, Goring *et al.* 1993, Nasrallah *et al.* 1994, Watanabe *et al.* 1997). These results indicate that *SLG* and/or *SRK* are necessary for the SI recognition reaction. In contrast, in some *S* haplotypes of *Brassica* species, deletion of *SLG* from the genome and mutation in the *SLG* sequence were observed, indicating that *SRK* solely functions in the SI recognition reaction at least in these *S* haplotypes (Okazaki *et al.* 1999, Suzuki *et al.* 2000, Suzuki *et al.* 2003).

The determination of the function of *SLG* and *SRK* by using gain-of-function experiments took more than 10 years of research after the isolation of the *SLG* and *SRK* genes. The difficulty in determining the function of *SLG* and *SRK* was due to the following two problems. One is that the transformation efficiency of *Brassica* species, especially *B. campestris*, was very low. Based on the screening of several cultivars of *B. campestris*, Takasaki *et al.* (1997) identified a suitable cultivar, Osome, for use as a plant material for transformation experiments. Another problem was that when the *SLG* or *SRK* genes were introduced into self-incompatible

Brassica species, co-suppression between the *SLG*/*SRK*-transgenes and the endogenous *SLG*/*SRK* genes occurred, and the transformants became self-compatible without acquiring new *S* haplotype specificity (Conner *et al.* 1997, Stahl *et al.* 1998, Takasaki *et al.* 1999). Takasaki *et al.* (1999, 2000) reported that this co-suppression depends on the sequence similarity between the *SLG*/*SRK*-transgenes and the endogenous *SLG*/*SRK* genes based on the following experiment. That is, when a class-I *SLG*⁹ gene was introduced into a self-incompatible cultivar, Osome, which is the *S* heterozygous plant derived from class-I (*S*⁵²) and class-II *S* haplotype (*S*⁶⁰), the expression of endogenous *SLG*⁵² was specifically suppressed in the transgenic plants, unlike that of *SLG*⁶⁰ (Takasaki *et al.* 1999). Thus, in order to avoid the co-suppression between transgene and endogenous gene, *SLG*⁹ and *SRK*⁹ of *B. campestris* were independently introduced into *S*⁶⁰ homozygous plants of *B. campestris* (Takasaki *et al.* 2000). The transgenic plants expressing the *SRK*⁹-transgene acquired the *S*⁹ haplotype specificity in the stigma, but not in the pollen. In contrast, the transgenic plants expressing the *SLG*⁹-transgene did not acquire the *S*⁹ haplotype specificity in the stigma nor in the pollen. These data clearly show that *SRK* is the sole determinant of the *S* haplotype specificity of the stigma (Takasaki *et al.* 2000). As previously described, *SLG* is also specifically expressed in the stigma tissues (Watanabe *et al.* 1994). What function does *SLG* have in the SI recognition reaction? When the number of seeds per pod obtained after crossing with *S*⁹ pollen was examined precisely, the plant harboring both *SRK*⁹ and *SLG*⁹-transgenes set few seeds (about 0.3 seeds per pod), a number comparable to that of the *S*⁹*S*⁶⁰ heterozygous plants (about 0.2 seeds per pod), and this number was significantly lower than that of the plants harboring the *SRK*⁹-transgene alone (about 1.9 seeds per pod) (Takasaki *et al.* 2000). These results suggested that *SLG* acts to promote the full manifestation of the SI response. Some self-compatible mutants, in which the *SLG* gene was deleted from the genome, were found to accumulate lower amounts of *SRK* protein compared with the self-incompatible plants, in spite of the similar level of *SRK* transcript (Dixit *et al.* 2000). In contrast, in an independent gain-of-function experiment using a related species, *B. napus*, the enhancing role of *SLG* was not observed (Cui *et al.* 2000, Silva *et al.* 2001). Studies on the difference of the *SLG* function in the SI response observed in these two experiments and on the more precise function of *SLG* need to be conducted in the future.

Identification and functional characterization of male *S* determinant

After the *SLG* and *SRK* genes were isolated and characterized, many SI researchers have tried to identify the male *S* determinant by using several strategies (reviewed in Hinata *et al.* 1993, Watanabe and Hinata 1999). The male *S* determinant is expected to exhibit the following four properties. First, the gene encoding the male *S* determinant must be lo-

cated at the *S* locus, suggesting that the gene should be close to the *SLG* and *SRK* genes. Secondly, the gene should be expressed before meiosis in the pollen mother cell, or expressed later in the anther tapetum cells, which are nurse cells investing the developing pollen grains, because the regulation of *Brassica* SI is sporophytic (Bateman 1955). Thirdly, the male *S* determinant gene must show allelic diversity among the *S* haplotypes, as observed in the stigma-specific genes, *SLG* and *SRK* (reviewed in Watanabe *et al.* 2001). Fourthly, the male *S* determinant should physically interact with *SLG* and/or *SRK* in an *S* haplotype-specific manner.

Two different approaches, biochemical and molecular genetic analyses, were adopted for the identification of the male *S* determinant. First, biochemical studies provided an important clue for the identification of the male *S* determinant. By using a band-shift assay in isoelectric focusing gels, a 7-kDa PCP (pollen coat protein), which was designated as PCP7 (later renamed PCP-A1), was identified as an *SLG*-interacting protein. However, its interaction was not *S* haplotype-specific and its gene was not linked to the *S* locus, indicating that PCP-A1 was not a real male *S* determinant (Doughty *et al.* 1993, Doughty *et al.* 1998). The PCP-A1 was a cysteine-rich basic protein, and a member of a large protein family, the PCP family (Doughty *et al.* 1998). A cDNA clone homologous to the PCP-A1 gene was also isolated from a cDNA library of immature anthers with polyclonal antiserum against the PCPs (Toriyama *et al.* 1998). By using a surface plasmon resonance sensor (BIAcore) along with chromatographic methods, many PCP-like molecules, which can interact with *SLG*, were identified in the proteins extracted from the pollen coat, though their interaction was not *S* haplotype-specific, like PCP-A1 (Takayama *et al.* 2000a). Furthermore, a pollination bioassay system monitoring the hydration of pollinated pollen grains was developed for the identification of the male *S* determinant from a pollen coat. By using this bioassay system, it was demonstrated that a small basic protein fraction (<10 kDa) containing PCP-A1 induced the SI response on the papilla cells in an *S* haplotype-specific manner, indicating that the male *S* determinant must be a PCP-A1-like peptide in the pollen coat (Stephenson *et al.* 1997).

Based on these biochemical experiments, two different molecular biological experiments were performed to identify the male *S* determinant. Firstly, in the *S*⁹ haplotype of *B. campestris*, a 76-kb genomic fragment containing the *SLG* and *SRK* genes was cloned into a P1-derived artificial chromosome (PAC) vector (Suzuki *et al.* 1997b). After complete sequencing of this fragment, 12 additional expressed genes were identified in this 76-kb *SLG*/*SRK* region (Suzuki *et al.* 1999, Watanabe *et al.* 1999). One of these genes, designated as *SP11* (*S* locus protein 11), which was located between the *SLG* and *SRK* genes, was specifically expressed in the anther tissues, and encoded a small basic protein, which had eight cysteine residues partially similar to the PCP-A1 protein (Suzuki *et al.* 1999). From *S*⁵² haplotype, a cDNA clone ho-

homologous to *S⁹-SP11* was also isolated by using *S⁹-SP11* as a probe (Takayama *et al.* 2000b). As a second approach, a fluorescent differential display (FDD) was used to compare anther cDNA populations between *S⁸* and *S¹²* haplotypes to identify the *S* haplotype-specific genes. Among 26 *S* haplotype-specific clones, one clone, *S⁸-SP11*, exhibited characteristics specific for *SP11* gene. With the information on the nucleotide sequence of *S⁸-SP11*, a cDNA clone homologous to *S⁸-SP11* was also isolated from *S¹²* haplotype by using RT-PCR. When the sequences of the deduced amino acids of these four clones (*S⁸-SP11*, *S⁹-SP11*, *S¹²-SP11* and *S⁵²-SP11*) were aligned, the hydrophobic region in the amino-terminal, which was expected to be a signal peptide, was highly conserved among the genes, though the region of the mature protein markedly diverged except for the cysteine residues (Takayama *et al.* 2000b). As determined in the *S⁹* haplotype, these genes were located upstream or downstream of the *SRK* gene in each *S* haplotype, indicating that the genes are alleles at the *S* locus (Suzuki *et al.* 1999, Takayama *et al.* 2000b, Watanabe *et al.* 2000a). The temporal and spatial expression analysis of the *SP11* gene clearly demonstrated that *S⁸-SP11* was sporophytically expressed in the tapetum cells at early developmental stages and also gametophytically in microspores at late developmental stages (Takayama *et al.* 2000b), explaining easily the sporophytic nature of *Brassica* SI. Independently, a group at Cornell University identified the same *S⁸-SP11* gene during the analysis of the *SLG/SRK* region of the *S⁸* haplotype of *B. campestris*, and it was designated as *SCR* (*S* locus cysteine-rich protein). By using *SCR⁸* as a probe, two genes orthologous to *SCR⁸* were isolated from a related species, *B. oleracea* (Schopfer *et al.* 1999).

In order to determine whether *SP11/SCR* is the male *S* determinant or not, gain-of-function experiments (Schopfer *et al.* 1999, Shiba *et al.* 2001) and a pollination bioassay (Takayama *et al.* 2000b) were performed. In the case of the gain-of-function experiments, when the *S⁸-* and *S⁹-SP11* genes were introduced into self-incompatible *B. campestris* (*S⁵²S⁶⁰* heterozygote), the pollen grains from transgenic plants expressing the *S⁸-* and *S⁹-SP11* transgene acquired the *S⁸-* and *S⁹-*haplotype-specificity, respectively (Shiba *et al.* 2001). A similar result was obtained in the case of *B. oleracea* (Schopfer *et al.* 1999). In the case of the pollination bioassay, which was developed by Stephenson *et al.* (1997), when papilla cells were pre-treated with the chemically synthesized and/or recombinant *S⁸-* and *S⁹-SP11* proteins, *S⁸-* and *S⁹-SP11* induced the SI response only when applied to the *S⁸-* and *S⁹-*stigma, respectively, and inhibited the cross-pollen hydration and germination (Takayama *et al.* 2000b, Takayama *et al.* 2001). All these results taken together suggest that *SP11/SCR* is the real male *S* determinant in SI.

Recently, the localization and translocation of SP11 during pollen development and pollination have been demonstrated by using immuno-electron microscopy. The SP11 protein was secreted from the tapetal cell into the anther locule as a cluster and translocated to the pollen surface at the

early developmental stage of the anther tissues. During the pollination process, SP11 was translocated from the pollen surface to the papilla cell, and then penetrated the cuticle layer of the papilla cell to diffuse across the pectin cellulose layer. Furthermore, SP11 could only penetrate the cuticle layer of the papilla cell in the presence of pollen grains, suggesting that some factors different from pollen grains must be necessary for SP11 to penetrate the papilla cell wall (Iwano *et al.* 2003).

In order to determine the allelic diversity of *SP11* among *S* haplotypes in *Brassica* species, several *SP11* allelic genes were isolated from the class-I *S* haplotypes by using RT-PCR with a primer designed from the conserved signal peptide region (Watanabe *et al.* 2000a, Suzuki *et al.* 1999, Takayama *et al.* 2000b, Sato *et al.* 2002). The amplified PCR product was found to be closely linked to the *SLG/SRK* genes, indicating that this PCR product was an allele of *SP11* (Watanabe *et al.* 2000a). Although the physical distances between *SLG/SRK* and *SP11* varied considerably among *S* haplotypes (Takayama *et al.* 2000b, Watanabe *et al.* 2000a, Suzuki *et al.* 2000, Kimura *et al.* 2002), the sizes of the *S* locus region of *B. campestris* were generally smaller than those of *B. oleracea*, probably because the multiple insertion events specifically occurred in the region of *B. oleracea* after speciation (Suzuki *et al.* 2000, Kimura *et al.* 2002). When the amino acid sequences of *SP11* were aligned, several characteristic features were observed. First, eight cysteine residues (C1 to C8) were conserved in the mature protein. Secondly, a glycine residue between C1 and C2 was conserved. Thirdly, one aromatic amino acid residue between C3 and C4 was conserved (Watanabe *et al.* 2000a, Sato *et al.* 2002). *SP11* genes derived from class-II *S* haplotypes were isolated and characterized in *Brassica* species (Shiba *et al.* 2002). These three characteristic features identified in class-I *S* haplotypes were also conserved in the *SP11* of class-II *S* haplotypes, indicating that these common primary structural features are important for the tertiary structure of *SP11* in general (Watanabe *et al.* 2000a, Shiba *et al.* 2002, Sato *et al.* 2002).

Recently, the SP11 protein has been purified from the pollen coat, and found to be present as a monomer stabilized with four disulphide bonds. By using the chemically synthesized SP11 protein, the disulphide linkage (C1-C8, C2-C5, C3-C6, C4-C7) of SP11 was determined (Takayama *et al.* 2001, Takayama and Isogai 2003). The synthesized form of SP11 was found to be biologically active by using a modified pollination bioassay system (Takayama *et al.* 2001).

The trans-specific mode of polymorphism of the *SP11* gene was observed from the phylogenetic tree (Watanabe *et al.* 2000a, Shiba *et al.* 2002, Sato *et al.* 2002), as was also the case with the *SLG* and *SRK* genes, as described above. The topology of the phylogenetic tree of *SP11* is very similar to that of *SLG* and the S domain of *SRK*, though the branching pattern of *SP11* is slightly different from that of *SRK*. Therefore, it appears that the three SI-related genes, *SLG*, *SRK* and *SP11*, have similar evolutionary histories (Watanabe *et al.*

2000a, Sato *et al.* 2002).

Interaction between male and female *S* determinants

As described above, the molecular features of the male (*SP11*) and female (*SRK*) *S* determinants were characterized, and the next question is to determine how the *SP11* molecule physically interacts with *SRK*. In order to analyze the interaction between *SP11* and *SRK*, ^{125}I -labeled *S*⁸-*SP11* (^{125}I -*S*⁸-*SP11*) displaying the native biological activity of *S*⁸-*SP11* was used to perform cross-linking experiments with stigmatic microsomal membranes. Two protein bands (120 and 65 kDa), which were cross-linked to ^{125}I -*S*⁸-*SP11*, were detected on SDS-PAGE gel. Based on the molecular masses of the protein bands and the immunoreactivity to specific antibodies to *SLG* and *SRK*-KD (kinase domain), the 120-kDa protein was considered to correspond to *SRK*⁸ and the 65-kDa protein probably corresponded to *SLG*⁸ (Takayama *et al.* 2001). However, because the truncated soluble protein of *SRK* (e*SRK*), which was derived from alternative splicing of the *SRK* gene (Giranton *et al.* 1995, Suzuki *et al.* 1996), and several other *SLG*-like proteins were expressed in stigma tissues (Suzuki *et al.* 1997c, Kai *et al.* 2001), it is necessary to determine whether the 65-kDa protein band corresponds to *SLG* or not. Furthermore, *S*⁸-*SP11* could specifically induce the autophosphorylation of *SRK*⁸, but not that of *S*⁹-*SP11*, clearly indicating that the *SP11* molecule solely can activate *SRK* in an *S* haplotype-specific manner (Takayama *et al.* 2001).

Independently, by using tagged versions of recombinant *SRK* (e*SRK*-FLAG) and *SCR* (*SCR*-Myc-His₆), the reactivity between *SCR* and *SRK* was investigated (Kachroo *et al.* 2001). In both the "pull-down" assay and ELISA experiments, e*SRK*₆-FLAG interacted more strongly with *SCR*₆-Myc-His₆ than with *SCR*₁₃-Myc-His₆, indicating that *SRK*-SD itself has an affinity to *SP11* (Kachroo *et al.* 2001), though no interaction between *SP11* and *SRK*-SD expressed in silkworm cells was observed (Takayama *et al.* 2001). Furthermore, e*SRK*-FLAG could interact with ca. 16-kDa PCP, suggesting that *SP11* might function as a dimer (Kachroo *et al.* 2001), which is in contrast to our data showing that *S*⁸-*SP11* with biological activity is present as a monomer (Takayama *et al.* 2001). It is difficult to explain at present the discrepancies in the data derived from different laboratories because of the variations in the materials and methods used in these experiments. However, these should be clarified in the future.

Signaling cascade downstream of *SP11*-*SRK* recognition

To date, three different molecules, which interact with the kinase domain (*SRK*-KD) and/or transmembrane domain of *SRK* (*SRK*-TM), have been identified by using different methodologies. These molecules are expected to offer important clues for understanding the *SI* downstream signaling pathway(s). By using a yeast two-hybrid system

with *SRK*-KD as bait, two kinds of proteins (*THL1*/*THL2* and *ARC1*) were isolated and characterized (Bower *et al.* 1996, Gu *et al.* 1998). The *ARC1* (arm-repeat-containing protein 1) protein bound to *SRK*-KD with a phosphorylation-dependent manner (Gu *et al.* 1998). Furthermore, when the expression of the *ARC1* gene was suppressed by its antisense gene, the transgenic plants became partially self-compatible, indicating that *ARC1* is a positive effector of the *SI* signaling (Stone *et al.* 1999). Recently, it has been shown that *ARC1* had displayed an E3 ubiquitin ligase activity, indicating that *ARC1* promotes the ubiquitination and proteasomal degradation of some compatibility factors in the pistil, which in turn leads to pollen rejection (Stone *et al.* 2003). In contrast, *THL1*/*THL2* (thioredoxin-h-1 and 2) proteins were found to interact with a conserved cysteine residue at *SRK*-TM in a phosphorylation-independent manner (Bower *et al.* 1996, Muzzurco *et al.* 2001). *In vitro* phosphorylation experiments showed that *SRK* was negatively regulated by *THL1* and activated by pollen coat proteins (presumably *SP11*) (Cabrillac *et al.* 2001). As another strategy, by using recombinant *SRK*-KD and [γ - ^{32}P]ATP, a phosphorylation-positive clone designated as *Bc*-NDPK-III (homologous to nucleoside diphosphate kinase) was isolated from the stigma cDNA library. Based on *in vitro* phosphorylation experiments, *SRK*-KD was found to be phosphorylated by *Bc*-NDPK (Matsushita *et al.* 2002). It is important to determine the precise roles of these interactive molecules in the *SI* signaling cascade.

Analyses of self-compatible lines, whose mutated genes are not linked to the *S* locus, are important for understanding the signaling cascade downstream of *SP11*-*SRK* interaction. One well-studied self-compatible line is the *B. campestris* var. *yellow sarson*, which was derived from a naturalized population in India. The compatibility of this line was found to be regulated by a recessive gene, designated as *m* gene (Hinata and Okazaki 1986). The *M* locus was independent of and epistatic to the *S* locus, and the *S*⁸*S*⁸*mm* plants exhibited a complete loss of self-incompatibility response in the stigma, but not in the pollen, suggesting that the *M* gene encodes a key effector working downstream of *SRK*. It was once suggested that the *M* gene encoded an aquaporin-like protein, *MOD* (Ikeda *et al.* 1997). However, recent molecular analyses on the *MOD* gene of several self-incompatible lines demonstrated that *MOD* was not the real *M* gene (Fukai *et al.* 2001). A schematic model of the recognition reaction in *Brassica* *SI* is presented in Fig. 2.

Dominance relationships on the stigma and pollen

To date, more than 30 and 50 *S* haplotypes have been identified in *B. campestris* (Nou *et al.* 1993) and in *B. oleracea* (Ockendon 2000), respectively. Because the *Brassica* *SI* system is sporophytically controlled, the *SI* phenotype of the stigma and pollen of a plant is determined by whether the two *S* haplotypes it carries are co-dominant or exhibit a dominant/recessive relationship (Thompson and Taylor 1966,

Fig. 2. Schematic model for self-pollen recognition in *Brassica* species. Three highly polymorphic SI genes, *SLG*, *SRK* and *SP11*, are located at the *S* locus. *SP11* is predominantly expressed in the tapetum cells of anther locules, and accumulates on the pollen surface during pollen maturation. Upon self-pollination, *SP11* molecules penetrate the papilla cell wall and interact with the receptor complex containing *SRK* molecules. This interaction induces the autophosphorylation of the *SRK* molecule. The activated *SRK* can phosphorylate the *ARC1* molecule. After the subsequent signal transduction, which is not defined, the rejection of the self-pollen occurs. The ligand-receptor interaction and receptor activation occur in an *S* haplotype-specific manner.

Hatakeyama *et al.* 1998b). The characteristics of the dominance relationships in *Brassica* species include: (i) common co-dominant relationships; (ii) dominant/recessive relationships occurring more frequently in pollen than in stigma; (iii) dominant/recessive relationships differing between pollen and stigma; (iv) non-linear dominant/recessive relationships (Thompson and Taylor 1966, Hatakeyama *et al.* 1998b).

In the case of the dominance relationship on the stigma side, the regulating molecule was determined by analyzing the transformants carrying the *SRK* gene. The pollination experiments and detection of transcripts of *SRK* suggested that the dominance relationships between *S* haplotypes in the stigma were determined by *SRK* itself, but not by the relative transcript level (Hatakeyama *et al.* 2001).

As for the dominance relationship on the pollen side, it was found that class-I *S* haplotypes are generally dominant over class-II *S* haplotypes (Hatakeyama *et al.* 1998b). Comparison of the sequences of *SLG*, *SRK* and *SP11* between class-I and class-II *S* haplotypes, showed that the sequences in each of the three genes were highly different between classes (Hatakeyama *et al.* 1998b, Shiba *et al.* 2002). Based on molecular cloning of the male *S* determinant, *SP11*, from class-I and class-II *S* haplotypes (Schopfer *et al.* 1999, Takayama *et al.* 2000, Watanabe *et al.* 2000a, Shiba *et al.* 2002), it is considered that the correlation between the classification of *S* haplotypes and sequence similarity of *SLG* and *SRK* results from the co-evolution between male (*SP11*) and female (*SRK*) *S* determinant genes (Watanabe *et al.* 2000a, Sato *et al.* 2002).

Recent RNA gel blot and *in situ* hybridization analyses of the *SP11* gene in the *S* heterozygotes with a pair of class-I and class-II *S* haplotypes have shown that the expression of class-I *SP11* was predominantly observed, while that of class-II *SP11* was completely repressed, suggesting that the dominance relationships between class-I and class-II *S* haplotypes on the pollen side are determined by the expression level of *SP11* (Shiba *et al.* 2002, Kakizaki *et al.* 2003). Similar phenomena were observed in another cruciferous *SI* plant, *Arabidopsis lyrata* (Kusaba *et al.* 2002).

Within four class-II *S* haplotypes of *B. campestris*, a linear dominance relationship ($S^{44} > S^{60} > S^{40} > S^{29}$) was observed on the pollen side. In all the six combinations of *S* heterozygotes derived from these four class-II *S* haplotypes, *SP11* from the dominant *S* haplotype was predominantly expressed, while *SP11* from the recessive *S* haplotype was always repressed. Therefore, the linear dominance relationship within class-II *S* haplotypes on the pollen side is also regulated by the RNA level of the *SP11* gene. Interestingly, S^{60} and S^{40} haplotypes can be both dominant and recessive depending on their *S* haplotype partner in the heterozygote, indicating that these linear dominance relationships are apparently reversible epigenetic phenomena (Kakizaki *et al.* 2003). More precise molecular mechanisms of expression of dominance relationships in both stigma and pollen remain to be elucidated in the future.

Future perspectives—from *Brassica* to *Arabidopsis*

During the last decade, molecular genetic and biochem-

ical analyses have revealed that the male and the female *S* determinants of *Brassica* SI are *SP11* and *SRK*, respectively. As described above, many problems still remain to be solved to fully understand the recognition reaction of *Brassica* SI. In order to elucidate the mechanism(s) controlling this function, the use of a cruciferous self-compatible plant, *A. thaliana*, is considered to be very important, because the genomic sequence of *A. thaliana* has already been determined (The Arabidopsis Genome Initiative 2000). In the genomic sequence of *A. thaliana*, the region homeologous to the *Brassica* *S* locus has been identified in the *ETR1* flanking region on chromosome I. In this region of *A. thaliana*, however putative genes orthologous to the *Brassica* SI genes, *SLG*, *SRK* and *SP11*, have not been found (Conner *et al.* 1998, Takada *et al.* 2001). Recently, it has been reported that *SRK* and *SCR* orthologous genes were mapped on an *ARK3* region in chromosome IV of *A. thaliana* by comparison with the *S* locus region of *A. lyrata*, a self-incompatible species closely related to *A. thaliana*. In *A. thaliana*, the *SRK* and *SCR* orthologs encode a nonfunctional protein, whereas *A. lyrata* harbors the functional *SRK* and *SCR* genes, suggesting that the self-compatibility of *A. thaliana* might be due to the inactivation of the *S* locus genes by mutation (Kusaba *et al.* 2001). Based on these data, it was expected that the introduction of genes encoding both male and female *S* determinants (*SP11/SCR* and *SRK*) would lead to a change from the self-compatible *A. thaliana* to a self-incompatible one. Actually, by using the genomic clone of the *SLG/SRK* region of *Brassica* species, *A. thaliana* was transformed. However, the transformants did not exhibit the SI phenotype (Bi *et al.* 2000). In contrast, when *A. thaliana* was transformed with *SRK* and *SCR* derived from a closely related species, *A. lyrata*, the transformants partially exhibited a weak SI phenotype, suggesting that the downstream signaling cascade leading to the rejection of self-pollen is maintained in *A. thaliana* (Nasrallah *et al.* 2002). If *A. thaliana* successfully acquires the strong SI phenotype, the transformants could become very informative materials for the analysis of the signaling cascade of *Brassica* SI (The Arabidopsis Genome Initiative 2000, Nasrallah *et al.* 2002).

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