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Molecular Biology of Self-incompatibility in *Brassica* Species

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Abstract

Many angiosperm plants express self-incompatibility (SI), through which they can recognize self-pollen and restrict fertilization to non-self-pollen. In species of *Brassica*, SI is sporophytically expressed, regulated by a single locus, *S*, with multiple alleles. Two stigma-specific genes, *SLG* and *SRK*, both of which locate at the *S* locus, are believed to play a role in the recognition reaction on the stigma side. Reviewed here are findings about *SLG* and *SRK* genes, the molecular characterization of *S*-multigene family, the genomic structure of *S* locus, and some aspects on signal transfer by the proteins encoded by these genes.

1. Introduction

Many flowering plants have a self-incompatibility (SI) system, which permits them to avoid self-fertilization. SI is defined as the inability of a fertile hermaphrodite plant to produce zygotes after self-pollination, and is a genetically controlled phenomenon (de Nettancourt, 1977). Two forms of SI, heteromorphic and homomorphic, are distinguished on the basis of floral morphology. Primula, buckwheat, star fruit, and others are known to have heteromorphic SI. Homomorphic SI includes sporophytic and gemetophytic SI, which are distinguished by the expression of *S* genes. In sporophytic SI, the behavior of pollen tubes is determined by the genotype of sporophyte from which the pollen was produced. Plants in the Brassicaceae, Convolvulaceae, and other families are known to have sporophytic SI. In contrast, plants in the Solanaceae, Rosaceae, and Papaveraceae, and other families have gemetophytic SI. In this type of SI, the behavior of a pollen tube is determined by the *S* alleles it itself contains. A number of economically important vegetables are included in the Brassicaceae (*Brassica oleracea*, *B. campestris* (syn. *rapa*), *Raphanus sativus* and others), and SI permits the maintenance of some agronomicaly valuable hybrid variety seeds.

A few short review articles on this subject, have been published recently (de Nettancourt, 1997; Nasrallah, 1997; Suzuki *et al.*, 1997d; Charlesworth and Awadalla, 1998; McCubbin and Kao, 1999; Kusaba and Nishio, 1999). These works promote a general understanding of this field.

2. Identification and characterization of *SLG* and *SRK*

SI in Brassicaceae is controlled by a sporophytic multiple allelic system at a single locus, *S* (Bateman, 1955; Thompson and Taylor, 1966; Okazaki and Hinata, 1984; Nou *et al.*, 1991; Nou *et al.*, 1993a, b). The numbers of segregating *S* alleles have been estimated by several studies (Nou *et al.*, 1991; Nou *et al.*, 1993a, b). Generally speaking, populations contain about 30 or more *S*. Nou *et al.*, (1993a) estimated that there are more than 100 *S* alleles in *B. campestris* throughout the world. In the sporophytic system, the behavior of pollen tubes is determined by the genotype of the sporophyte that produced the pollen. Therefore, the phenotype of the pollen and the stigma of heterozygous plants depends upon complex dominant/recessive allelic interactions (Thompson and Taylor, 1966; Hatakeyama *et al.*, 1998a; **Fig. 1**). Among the characteristic features of dominance relationships in these species are: (i) a higher frequency of co-dominant relationships; (ii) a higher frequency of domi-

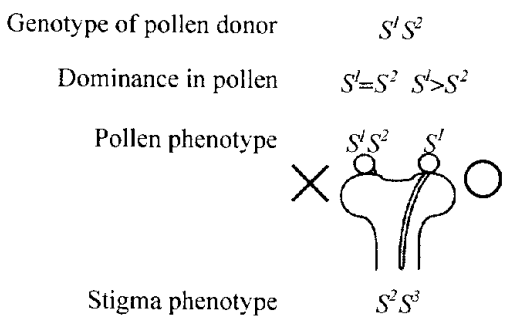

Fig. 1 A schematic illustration of the pollen tube behavior in sporophytic SI system upon the arrival of a pollen grain produced by an S^1S^2 plant on S^2S^3 stigma, in which S^2 and S^3 are co-dominant. If S^1 and S^2 are co-dominant in the pollen parent (left side), the phenotype of pollen is S^1S^2, and the pollen grain is rejected by the S^2S^3 stigma due to recognition of S^2. If S^1 is dominant to S^2 in the pollen (right side), the phenotype of the pollen becomes S^1, and the pollen tube penetrates papilla cells of the stigma.

nant/recessive relationships in the pollen than in the stigma; (iii) differences between stigma and pollen in dominant/recessive relationships; and (iv) higher incidence of non-linear dominance relationships in the stigma than in the pollen.

Identification of *S*-specific antigens in stigma made possible the molecular level dissection of the biology and genetics of SI in *Brassica* species. Isoelectric focusing (IEF) analysis of stigma proteins revealed that the *S* locus glycoproteins (SLGs) corresponding to respective *S* alleles had different pI values (Nishio and Hinata, 1977). These SLGs were shown to cosegregate with *S* alleles without exception (Hinata and Nishio, 1978; Nou *et al.*, 1991, Nou *et al.*, 1993a). SLGs are produced in stigmas a few days before flower anthesis with expression coincident with the expression of SI (Nishio and Hinata, 1977). SLG accumulates in the mature papilla cell wall, where inhibition of self-pollen tube development occurs (Kandasamy *et al.*, 1989; Kishi-Nishizawa *et al.*, 1990). Three SLGs were isolated from the stigmas of *B. campestris*, and partial amino acid sequences determined (Takayama *et al.*, 1987; Isogai *et al.*, 1987). Each sequence contains twelve conserved cysteine residues at the carboxyl terminal. No discernible differences were found in the *N*-glycosidic carbohydrate chains among these three SLGs (Takayama *et al.*, 1989), suggesting that *S* allele specificity is determined by the protein portions of SLGs (Takayama *et al.*, 1987; Isogai *et al.*, 1987).

Differential screening with *B. oleracea* led to the molecular cloning of *SLG* cDNA (Nasrallah *et al.*, 1985). The amino acid sequence deduced from *SLG* cDNA revealed a hydrophobic signal peptide at the amino terminal (Nasrallah *et al.*, 1987). Since these experiments, over thirty *SLG* clones have been isolated in *Brassica* species (Lalonde *et al.*, 1989; Trick and Flavell, 1989; Chen and Nasrallah, 1990; Scutt and Croy, 1992; Delorme *et al.*, 1995a; Yamakawa *et al.*, 1994; Watanabe *et al.*, 1994; Matsushita *et al.*, 1996; Kusaba *et al.*, 1997; Hatakeyama *et al.*, 1998b, c; Goring *et al.*, 1992a, b). The nucleotide sequences of the same *S* specificity (SLG^{24}) derived from different populations (Japan and Turkey) were found to be completely conserved in both coding and non-coding regions (Matsushita *et al.*, 1996). The various *SLG*s so far cloned have been classified into two groups based on sequence similarity. The first group contains pollen-dominant *SLGs* (Class I *SLG*), which have no intron (Nasrallah *et al.* 1988, Suzuki *et al.* 1997b), and the second contains pollen-recessive *SLG*s (Class II *SLG*), which have one small intron at the 3' end of *SLG* (Tantikanjana *et al.*, 1993; Hatakeyama *et al.*, 1998b). Amino acid sequence homology among SLGs within each class is about 78-98%, but that between classes falls to about 65%. Comparison of phylogenetic relationships among *SLG*s suggests that allelic differentiation occurred before species divergence within the genus (Dwyer *et al.*, 1991), over the course of tens of millions of years (Hinata *et al.*, 1995; Uyenoyama, 1995).

The physical localization of the *SLG* in the chromosome of *B. campestris* was visualized by multi-color fluorescent *in situ* hybridization. The *SLG* gene is localized at the interstitial region close to the end of the chromosome (Iwano *et al.*, 1998).

Walker and Zhang, (1990) pointed out that the amino acid sequence of the extracellular domain of a serine/threonine type putative transmembrane protein kinase from maize had high homology to *SLG*. Subsequently, a gene encoding kinase, whose extracellular domain was highly homologous to *SLG*, was isolated from *B. oleracea*, and named *SRK* (*S*-receptor kinase; Stein *et al.*, 1991). Like *SLG*, *SRK* is tightly linked to the *S* locus (Stein *et al.*, 1991). Its extracellular domain (S domain) is connected via a single-pass transmembrane domain to a protein kinase catalytic center. To date, over ten sets of *SLG* and *SRK* genes from different *S* alleles of *Brassica* species have been isolated (Stein *et al.*, 1991; Chen and Nasrallah, 1990, Kumar and Trick, 1994; Delorme *et al.*, 1995a; Watanabe *et al.*, 1994; Yamakawa *et al.*, 1995; Suzuki *et al.*, 1995; Hatakeyama *et al.*, 1998b, c; Goring and Rothstein, 1992; Glavin *et al.*, 1994). Nucleotide sequence homology between *SLG* and the S domain of *SRK* derived from the same *S* allele is about 90%, and

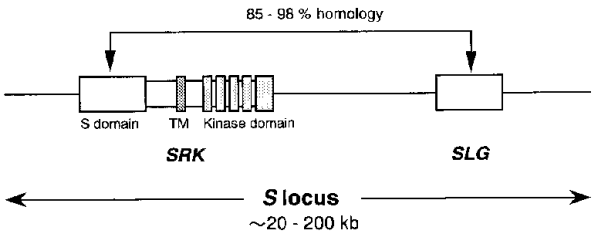

Fig. 2 Molecular structure of the chromosomal region containing the *SLG* and *SRK*. TM: transmembrane domain.

some cases exceeds 98% (Watanabe *et al.*, 1994; Hatakeyama *et al.*, 1998c; **Fig. 2**). The product of *SRK*, expressed as a fusion protein in *E. coli*, was found to be a functional protein kinase, which was able to autophosphorylate serine and threonine residues (Goring and Rothstein, 1992; Stein and Nasrallah, 1993). The SRK protein was shown to be a glycoprotein targeted to the plasma membrane (Delorme *et al.*, 1995a; Stein *et al.*, 1996). The transcript of *SRK* was mainly detected in stigma tissue with expression among developmental stages similar to that of *SLG* (Glavin *et al.*, 1994; Stein *et al.*, 1996). Even in stigma tissue, the transcript of *SRK* was extremely low relative to *SLG* (Stein *et al.*, 1991; Watanabe *et al.*, 1994; Glavin *et al.*, 1994; Delorme *et al.*, 1995a). This lower level expression of *SRK* compared to *SLG* correlates with transcription in the antisense direction of the promoter, exon I, and intron I of *SRK* (Cock *et al.*, 1997).

All *SRK* genes so far analyzed have comprised seven exons, with the first exon encoding the S domain (**Fig. 2**). Genomic clones of *SRK* show an in-frame stop codon near the 5'-splicing site of the first intron in the *SRK* genomic clone, suggesting that *SRK* directs the synthesis of several transcripts (Stein *et al.*, 1991; Kumar and Trick, 1994; Suzuki *et al.*, 1995; Suzuki *et al.*, 1996; Delorme *et al.*, 1995a). These transcripts are apparently generated through a combination of alternative splicing and polyadenylation signals (Stein *et al.*, 1991; Suzuki *et al.*, 1996; Giranton *et al.*, 1995). A truncated *SRK*, derived from the S domain, has been detected as a protein and as an RT-PCR product (Giranton *et al.*, 1995).

Because the *S* locus comprises multiple genes within one segregational unit as described below, an "*S* allele" is referred to as an "*S* haplotype" (Nasrallah and Nasrallah, 1993).

3. Functional analysis of *SLG* and *SRK*

Analysis of self-compatible mutants is an important strategy for elucidating the role of SLG and SRK in the SI recognition reaction. A single suppressor gene in a self-compatible mutant in *B. oleracea* was shown to have caused the reduction of *SLG* content, while the level of transcript of *SRK* was normal (Nasrallah *et al.*, 1992). An *SRK* isolated from self-compatible *B. napus* showed a 1-bp deletion toward the 3' end of the S domain, which would appear to cause premature termination of translation and the production of a truncated *SRK* (Goring *et al.*, 1993); this finding suggests that an active *SRK* might be required for the expression of SI. Analysis of genomic clones derived from a self-compatible *B. oleracea* that expressed *SRK* transcripts at undetectable levels but *SLG* at normal levels revealed the deletion of the first and the second exons of *B. oleracea* (Nasrallah *et al.*, 1994). In spontaneous self-compatible *B. campestris* var. *yellow sarson* (C636), self-compatibility (SC) was attributed mainly to a recessive epistatic gene (Watanabe *et al.*, 1997). In this strain, *SLG* transcripts were less abundant than that in self-incompatible strains and *SRK* transcripts were not detected, suggesting that the down-regulation of *SLG* and *SRK* may be related to SC.

Some transformation experiments with *SLG* or *SRK* have been conducted to elucidate their functional roles in SI. In a transgenic *B. campestris* with antisense *SLG* driven by the *SLG* promoter, transcripts of *SLG* and *SRK* decreased, and the transformant became self-compatible (Shiba *et al.*, 1995). When self-incompatible *B. campestris* was transformed with *SLG* derived from *B. campestris*, the SI phenotype of transformants changed to self-compatible as a result of alteration of SI phenotype of the stigma part, but not of the pollen part. This alteration of SI phenotype reflected co-suppression between the *SLG*-transgene and the endogenous *SLG* gene (Takasaki *et al.*, 1999). An *SLG* gene of self-incompatible *B. campestris* under control of a tapetum-specific promoter was introduced into self-compatible *B. napus*. A pollination test indicated that the pollen of the transgenic *B. napus* did not gain the SI phenotype (Sasaki *et al.*, 1998). Self-incompatible *B. napus* transformed with an inactive copy of *SRK* gene became self-compatible due to co-suppression and dominant-negative effects (Stahl *et al.*, 1998). The transgenes led to a dramatic reduction in the expression of the endogenous *S* locus and related genes indicating homology-dependent silencing. The silencing of *SLG* and/or *SRK* genes in self-incompatible host plants resulted in the breakdown of SI (Conner *et al.*, 1997). In both cases, the change of *S* phenotype was only observed in stigma, but not in pollen. These experiments indicate that both *SLG* and *SRK* are involved in the recognition reaction of SI, though gain of *S*

gene phenotype has not been successfully accomplished through transformation with *SLG* and *SRK*.

4. Molecular characterization of *S* multigene family

Southern blot analysis of *Brassica* genome using *SLG* cDNA probe showed multiple bands with *S* haplotype-associated restriction site polymorphism (Nasrallah *et al.*, 1985; Nasrallah *et al.*, 1988; Nou *et al.*, 1993b), indicating that many clones homologous to *SLG* and/or *SRK* exist in *Brassica* genome. Some of these hybridized bands have been isolated and characterized as *SLG*-like and *SRK*-like genes from *B. campestris*, *B. oleracea*, and *Arabidopsis thaliana*, indicating the existence of a large *S* multigene family (Dwyer *et al.*, 1989; Kumar and Trick, 1993; Dwyer *et al.*, 1994; Suzuki *et al.*, 1995). In the *S* multigene family, *SLR1* (*S* locus related gene 1; Isogai *et al.*, 1988; Isogai *et al.*, 1991; Lalonde *et al.*, 1989; Trick and Flavell, 1989; Trick, 1990; Yamakawa *et al.*, 1993; Watanabe *et al.*, 1998), *SLR2* (*S* locus related gene 2; Scutt *et al.*, 1990; Boyes *et al.*, 1991; Tantikanjana *et al.*, 1996), and *SLR3* (*S* locus related gene 3; Cock *et al.*, 1995) are not linked to the *S* locus (Lalonde *et al.*, 1989; Boyes *et al.*, 1991; Watanabe *et al.*, 1992; Cock *et al.*, 1995). *SLR1*, *SLR2*, and *SLR3* do not participate in the self- vs. non-self recognition events, at least not directly. *SLR1* has shown few alleles and low variation of the nucleotide sequence among alleles (Watanabe *et al.*, 1992; Hinata *et al.*, 1995; Watanabe *et al.*, 1998). Observation of *SLR1* antisense transgenic *B. napus* (Franklin *et al.*, 1996) revealed that antisense *SLR1* reduced adhesion between pollen and stigma, and indicated that SLR1 played a role in pollen-stigma adhesion (Luu *et al.*, 1997). *SLR2* is highly homologous to Class II *SLG* genes. The function of *SLR2* has not yet been determined: a mutant plant having low expression of *SLR2* showed normal self-incompatible and cross-compatible phenotype (Tantikanjana *et al.*, 1996).

One of members of the *S* multigene family, *SFR2* (*S* gene family receptor 2) was isolated and characterized in *B. oleracea*. This *SRK*-like was highly induced by wounding and bacterial infection, suggesting a relationship between this gene and plant defense reaction (Pastuglia *et al.*, 1997a). Another member, *ARK1* (*Arabidopsis* receptor kinase 1), is expressed in leaves and floral buds; it is possibly related to processes such as cell expansion or plant growth (Tobias *et al.*, 1992; Tobias and Nasrallah, 1996).

These observations indicate that members of *S* multigene family might play various roles in signal transduction in Brassicaceae.

5. Genomic structure of *S* locus

The extensive genomic regions spanned by the *S* locus have been analyzed in some *S* haplotypes. These analyses have revealed that several genes exist in the flanking region of *SLG* and *SRK* genes.

Comparison of the promoter regions of *SLG* and *SRK* genes revealed that Boxes I to V, which presumably function in stigma-specific expression, were highly conserved among the genes (Delorme *et al.*, 1995; Hatakeyama *et al.*, 1998b; Suzuki *et al.*, 1995; Stein *et al.*, 1996; Dzelzkalns *et al.*, 1993). In S^9 homozygote of *B. campestris*, a region of about 3.9-kb, containing the 5' non-coding and coding regions, has been shown to be completely identical between SLG^9 and SRK^9. This identity may suggest the recent occurrence of gene conversion (Watanabe *et al.*, 1994; Suzuki *et al.*, 1997a).

Some members of the *S* multigene family are closely linked to the *S* locus (Oldknow and Trick, 1995; Suzuki *et al.*, 1997b). In *B. campestris*, the physical distance between *SLG*, *SRK* and three *S*-related genes (*BcRK1*, *BcRL1*, and *BcSL1*), all linked to the *S* locus, was estimated to be less than 610 kb. The observation that the three *S*-related genes are expressed in both floral and vegetative tissues (Suzuki *et al.*, 1997b) suggests that they are not directly correlated with the recognition reaction of SI.

Under the assumption that the pollen *S* determinant of the SI recognition reaction is linked to the *S* locus, identification of the extent of the genomic region that co-segregates with the *S* locus may aid in its isolation. The physical distance between *SLG* and *SRK*, both of which co-segregate with the *S* locus, has been estimated as less than 200 kb in *B. oleracea* (Boyes and Nasrallah, 1993), less than 20 kb in *B. campestris* (Boyes *et al.*, 1997; Suzuki *et al.*, 1999), 25 kb in self-incompatible *B. napus* (Yu *et al.*, 1996), in which the *S* locus was derived from *B. campestris* through introgression. The variation among these estimates suggests that the size of the *S* locus may vary among plant species. Using P1-derived artificial chromosome (PAC) vector, Suzuki *et al.*, (1997c) directly cloned an 80-kb *Mlu*I genomic fragment containing both *SLG* and *SRK* genes of a *B. campestris* S^9 homozygote (**Fig. 3**).

In addition, several genes whose nucleotide sequences show no homology to those of *SLG* and *SRK* are located in the flanking regions of *SLG* and *SRK*. *SLA* (*S* locus anther gene), identified in an S^2 homozygote of *B. oleracea*, is located in the 3'-

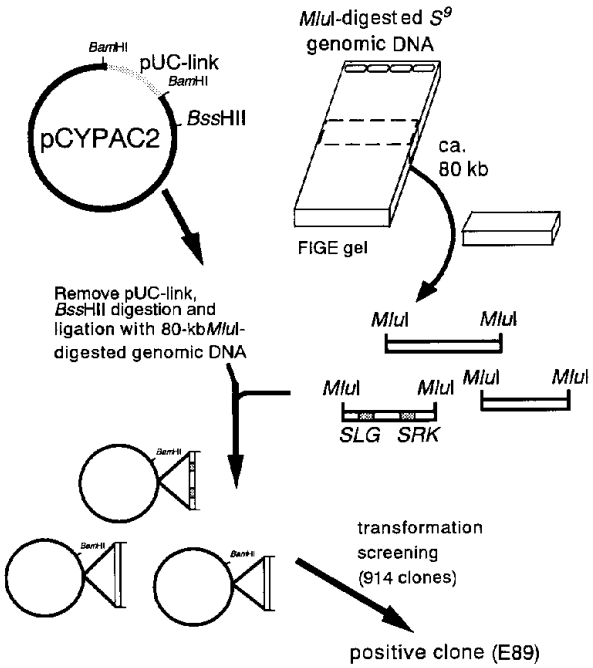

Fig. 3 Strategy for the cloning of an *Mlu*I genomic fragment which contains both SLG^9 and SRK^9.

flanking region of SLG^2 and expressed specifically in the anther (Boyes and Nasrallah, 1995). However, the identification in an self-incompatible *B. oleracea* of a non-functional *SLA*, disrupted by a retrotransposon, indicates that *SLA* is not required for the SI response (Pastuglia *et al.*, 1997b). In self-incompatible *B. napus*, two genes, *SLL1* (*S*-locus linked gene 1) and *SLL2* (*S*-locus liked gene 2), expressed in anther, were located between the *SLG* and *SRK* genes. However, *SLL1* did not show *S*-haplotype specificity and *SLL2* was also expressed in stigmas (Yu *et al.*, 1996). In an S^8 haplotype of *B. campestris*, two nonpolymorphic and vegetatively expressed sequences, *298* and *299*, were located in the 3'-flanking region of the SLG^8. It was found that sequence *299* encoded the *SLL2* gene and the *298* encoded *ClpP* (Clp protease) homologue (Boyes *et al.*, 1997; Conner *et al.*, 1998; Letham and Nasrallah, 1998). Recently, the *Mlu*I genomic fragment (*SLG/SRK* region) of the S^9 homozygote of *B. campestris* was screened for expressed sequences (Watanabe *et al.*, 1999; Suzuki *et al.*, 1999). This fragment contained 12 genes in addition to SLG^9 and SRK^9. This estimate of gene density (1 gene/5.4 kb) suggests that the *S* locus is embedded in a gene-rich region of the genome. Among the 12 genes detected, two (*SAE1*: *S* locus anther expressed gene 1 and *SP11*: *S* locus protein 11) were specifically expressed in anther tissues, and were located downstream of the SLG^9 or SRK^9 genes (Watanabe *et al.*, 1999; Suzuki *et al.*, 1999). The extent of allelic polymorphism and the function of *SAE1* and *SP11* are now under investigation.

For an S^8 haplotype of *B. campestris*, a 100-kb region spanning the *S* locus was mapped with several cDNA and genomic DNA clones of *Arabidopsis*. Comparative mapping between the *S* locus region of *Brassica* and the homoeologous region in *Arabidopsis* revealed that no sequences similar to the *Brassica S* locus in the *Arabidopsis* genome (Conner *et al.*, 1998).

6. Aspects on signal transduction

By analogy with animal growth factors, one may imagine that *SRK* accepts a signal from pollen and transduces the signal into papilla cells via a protein phosphorylation cascade, though the putative cascade triggered in the papilla cell is not clear. Recently, proteins interacting with SRK^{910} kinase domain have been screened by Goring's group using a yeast two-hybrid system. Two different kinds of cDNA clones were isolated and characterized. One of them included two thioredoxin-h-like clones, *THL-1* and *THL-2*. These clones specifically interacted with the kinase domain of SRK^{910}. *THL-1* was expressed in a variety of tissues, but *THL-2* preferentially expressed in floral tissues. Thioredoxin may possibly be one of the effector molecules in the signal cascade of SI (Bower *et al.*, 1996). Another cDNA clone contained the *ARC1* (Arm Repeat Containing) gene. The ARC1 specifically interacted with the kinase domain of SRK, but not with the kinase domains taken from a different kind of *Arabidopsis* receptor-like kinases. The interaction was phosphorylation dependent (Gu *et al.*, 1998). Another strategy for the elucidation of the signal transduction cascade is the analysis of self-compatible mutants caused by genes other than the *S* gene. Recently, the self-compatible *mod* (renamed from *m*) locus, which is not linked to the *S* locus, was dissected with molecular techniques. The self-compatible phenotype was associated with the absence of transcripts encoded by an aquaporin-related gene. This may suggest that a water channel is required for the SI response of *Brassica* species (Ikeda *et al.*, 1997).

Many studies seeking to identify the pollen components involved in SI have been conducted recently. Several pollen coat proteins, derived from tapetum cells, have isolated and characterized. One of the pollen substances, designated PCP7 (Pollen coat protein 7), is able to interact with SLGs. The *PCP* genes formed a large multigene family, but showed no band linked to the *S* locus (Stanchev *et al.*, 1996). By using polyclonal antiserum raised against the extracellular pollen proteins, cDNA clones homologous to *PCP* have also been isolated

from a cDNA library derived from immature anthers (Toriyama *et al.*, 1998). Recently, the effects of application of PCP-A (renamed from PCP7) to the stigma surface were examined using a bioassay system. When the "self" PCP-A fraction was given, compatible cross-pollination was prevented, while a "cross" PCP-A fraction could induce the germination and growth of self-pollen. This suggests that a member of the PCP-A protein family may be a determinant at the pollen side in the *Brassica* SI system (Stephenson *et al.*, 1997; Doughty *et al.*, 1998).

Further analysis of the recognition reaction of SI will be conducted through a combination of a broad spectrum of approaches including genetics, genomics, molecular biology, biochemistry, and biophysics.

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