Molecular Genetic Aspects of Self-incompatibility in Brassicaceae

Hee-Jeong Jung^{1†}, Nasar Uddin Ahmed^{1†}, Jong-In Park¹, Mi-Young Chung², Yong-Gu Cho³, Ill-Sup Nou^{1*}

¹Department of Horticulture, Sunchon National University, 255 Jungang-ro, Suncheon, Jeonnam 540-950, Republic of Korea

²Department of Agricultural Education, Sunchon National University, 255 Jungang-ro, Suncheon, Jeonnam 540-950, Republic of Korea

³Department of Crop Science, Chungbuk National University, 52 Naesudongro, Heungdokgu, Cheongju 361-763, Republic of Korea

ABSTRACT Molecular genetic studies of self-incompatibility (SI) are the most accentuating part in the way of advancement of reproductive mechanisms in flowering plants. In the Brassicaceae plants, self-incompatibility has been mapped genetically to a single chromosomal location where several closely linked genes have been identified. Recently, various studies have provided a novel insight into the basis of specificity in the *S*-receptor kinase (*SRK*) and *S*-locus protein 11 or *S*-locus Cysteine-rich (*SPI1/SCR*) interaction, the nature of the signaling cascade that culminates in the inhibition of 'self' pollen, and the physiological and morphological changes that are associated with transitions between the outbreeding and inbreeding modes of mating in the Brassicaceae. In this review, we discuss the current view of the molecular genetic aspects of the self-incompatibility in Brassicaceae.

Keywords Brassicaceae, Genetics, Recognition mechanism, Self-incompatibility

INTRODUCTION

Self-pollination occurs easily in hermaphrodite flowers, and leads to inbreeding depression and decreased genetic variation; therefore plants have evolved several mechanisms to avoid self-pollination. Self-incompatibility (SI) is defined as the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination, which is surely the most elegant pollination system (de Nettancourt 2001). SI is a physiological barrier making it difficult or impossible for a flower to fertilize itself even though it may be abundantly pollinated with its own pollen and it is the commonest way in which plants avoid self-fertilization. SI involves the ability of a plant to discriminate between its own pollen grains and those of another plant and only allow pollen from a different plant to grow and fertilize the ovules. In relation to biological events, incompatibility and sterility are clearly different. Sterility is caused by non-functional male and/or female components, whereas in incompatibility there is a lack of seed formation in a specific male and

female combination, both of which are functional. Because the final phenotypes in incompatibility and sterility are quite similar, this phenomenon was originally termed self-sterility. Taking the initial letter of sterility, the genetic locus regulating SI was termed the *S*-locus, which is still used as the authorized locus name. It is classified into heteromorphic and homomorphic type with respect to flower morphology. The homomorphic self-incompatibility of some species is controlled by a single locus, and those of the other species are controlled by multiple loci. In all the homomorphic systems, self-incompatibility is controlled by a multiallelic locus called *S*-locus and pollen is rejected by pistils having the same alleles. Homomorphic self-incompatibility systems can be divided into two major classes: gametophytic and sporophytic, which are distinguished by the expression of *S*-locus genes (de Nettancourt 1977). In the sporophytic form of self-incompatibility, the stigma surface is the site of recognition and the proteins in the outer coat of the pollen grain are recognized. This pollen surface material is derived from the parent plant, the

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*Corresponding author Ill-Sup Nou, nis@sunchon.ac.kr, Tel: +82-61-750-3249, Fax: +82-61-750-3208

[†]These two authors equally contributed to this work.

sporophyte or spore-bearing plant, not from the pollen grain itself, hence the name of this system. Plants in the Brassicaceae, Convolvulaceae, and other families are known to have sporophytic self-incompatibility. For instances, pollen grains from the S^1S^2 anther are rejected on the S^1S^2 pistil due to matching alleles, however, the pollen grains from the S^1S^2 anther are fully compatible on an S^3S^4 pistil, and fertilization occurs.

In SI plants of this family, pollen will not develop on a stigma that expresses the same alleles as the pollen parent. The various naturally occurring, classically defined *S*-alleles that have been described in *Brassica* have been arranged in a dominance series based on their genetic behavior relative to other alleles in heterozygous plants (Thomson and Taylor 1966). Molecular analysis of the *S*-locus region shows that this locus is a complex locus spanning many kilobases and containing several physically linked transcriptional units that cosegregate perfectly with SI phenotype (Boyes *et al.* 1997; Casselman *et al.* 2000). A subset of genes within the *S*-locus complex (*S*-haplotype) is highly polymorphic as expected for genes involved in recognition, and specific combinations of allelic forms of each of these genes are thought to define different SI specificities. Thus, the *S*-locus may be viewed as a master recognition locus that encodes the function(s) required for the stigma to distinguish self-related from self-unrelated pollen. In other words, we are interested in *Brassica* SI from the viewpoint of both fundamental biology and agricultural applications. Thus, an understanding of the molecular mechanisms of SI in *Brassica* species is an important research target.

In this review article, we will summarize SI research in Brassicaceae plants, including molecular cloning of the male and female *S* determinants, epigenetic regulation of dominance relationships between *S*-alleles, and evolution of the SI system.

Genetics of Self-Incompatibility in Brassicaceae plants

Bateman (1955) deciphered the genetics of SI in the family Brassicaceae in the early 1950s. He described the control of SI by a single Mendelian locus, the *S* (Sterility) locus, which exists as multiple alleles or variants, each of which encodes a distinct mating specificity. As expected for a system in which new alleles have a reproductive

advantage and therefore will increase in frequency toward equilibrium, the number of *S*-locus alleles is usually large, being estimated at 22 in *Iberis* (Bateman 1955), 34 in *Raphanus* (Sampson 1957), 30 in *B. rapa* (Nou *et al.* 1993), and more than 50 in *Brassica oleracea* (Brace *et al.* 1994). The various naturally occurring, classically defined *S*-alleles that have been described in *Brassica* have been arranged in a dominance series based on their genetic behavior relative to other alleles in heterozygous plants (Thomson and Taylor 1966). A classical genetic analysis has grouped the *Brassica S* alleles into two categories based on their phenotypic effect on self-incompatibility characteristics. The first group of alleles (high-activity) is placed relatively high on the dominance scale and exhibits a strong self-incompatible phenotype in which an average of 0 to 10 pollen tubes develop per self-pollinated stigma. The second group of alleles (low-activity) demonstrates a weak or leaky self-incompatible phenotypic effect in which 10 to 30 pollen tubes develop per self-pollinated stigma and they are considered to be recessive (Nasrallah *et al.* 1991).

The *S*-locus holds a large number of alleles within a population by frequency-dependent selection (Wright 1939; Fisher 1958). This system involves self- or non-self-pollen recognition in the reproductive process. Several self-recognition mechanisms have been discovered in multiple plant lineages. They are classified into two types of pollen-side recognition, i.e., gametophytic (haploidy recognition) and sporophytic (diploidy recognition) self-incompatibility (Takayama and Isogai 2005). Self-recognition reaction requires two genes—one for the female (pistil) side and the other for the male (pollen) side—and allele-specific interaction between female and male molecules prevents self-fertilization to avoid inbreeding depression. Recombination between the two genes should be suppressed because recombination may disrupt the allele-specific self-recognition ability. Balancing selection on the *S*-locus increases the nucleotide diversity in regions around the two self-incompatibility-related genes. Sequence surveys of the *S*-locus region have been performed in *Brassica* species and *Prunus mume*, which employ sporophytic and gametophytic self-incompatibility systems, respectively (e.g., Boyes *et al.* 1997; Casselman *et al.* 2000; Entani *et al.* 2003; Fukai *et al.* 2003; Shiba *et al.* 2003). These studies have revealed extremely high nucleotide

diversity in 10 kb in *Brassica rapa* (Shiba *et al.* 2003) and in *P. mume* (Entani *et al.* 2003). It has been thought that high nucleotide variation may result from the suppression of recombination in the entire *S*-locus region.

SI recognition genes identified from stigma

Until now, several closely linked genes have been identified at the *S*-locus. Two of them, *SLG* (for *S*-locus glycoprotein; Nasrallah *et al.* 1985) and *SRK* (for *S*-locus receptor kinase; Stein *et al.* 1991), are thought to be involved in the perception of self-pollen by the stigma. Both genes are highly polymorphic, and both are expressed specifically at the surface of mature stigmas. Mutations in *SRK* (Goring *et al.* 1993; Nasrallah *et al.* 1994) and loss of *SLG* expression (Toriyama *et al.* 1991; Nasrallah *et al.* 1992; Shiba *et al.* 1995) have been associated with SI. *SLG* gene is about 1.3 kb in length and encodes a 55 kDa glycoprotein secreted into the papillar cell wall. Although the deduced amino acid sequence of *SLGs* are highly polymorphic, 12 conserved cysteine residues are found in common, suggesting the importance of the structure formed by the cysteine residues for the common function of the glycoprotein. There are several potential N-linked glycosylation sites, but only two conserved sites (Kusaba *et al.* 1997). The role played by *SLG* in the SI response started to be questioned after the characterization of self-incompatible plants which express a very low level of *SLG*, and, in particular, after the discovery of naturally self-compatible haplotypes which express a high level of *SLG* (Gaude *et al.* 1995). New observations suggest that *SRK*, rather than *SLG*, plays a key role in the SI reaction. The *SRK* gene encodes a membrane-associated protein. A number of pieces of evidence indicate that a functional *SRK* is required for SI response in *Brassica*. The predicted SRK protein consists of a potentially glycosylated extracellular domain (*S*-domain) that shares extensive sequence similarity with *SLG* (90%) within haplotypes and contains 12 cysteine residues found in all members of the *S* gene family in the Brassicaceae. This domain is joined via a single-pass transmembrane domain to a cytoplasmic region that has sequence similarity to protein kinases. Bacterially expressed SRK kinase domains show serine/threonine autophosphorylation activity (Goring and Rothstein 1992).

SRK may be related to plant proteins involved in defense against pathogens, another plant recognition system (Pastuglia *et al.* 1997a). Other approaches to studying the SI signaling cascade have used *Arabidopsis* species, because the whole genome sequence of it has been determined and other genetic tools (e.g. T-DNA tag lines, many ecotypes and microarray expression data) are available. To produce self-incompatible *A. thaliana* by transformation, two approaches have been used. Nasrallah and co-workers introduced *SP11* and *SRK* from self-incompatible *A. lyrata* into self-compatible *A. thaliana* (C24 ecotype) and the resulting transgenic *A. thaliana*, with both *SP11* and *SRK*, exhibited the SI trait (Nasarallah *et al.* 2002, 2004). Searches for SRK regulators led to the identification of several interacting proteins (Bower *et al.* 1996; Gu *et al.* 1998; Vanoosthuyse *et al.* 2003; Kakita *et al.* 2007b). Three of these, *ARC1*, *THL1*, and *MLPK*, have been shown to be involved in the SI response (Stone *et al.* 1999; Haffani *et al.* 2004; Murase *et al.* 2004). *THL1* has been demonstrated to negatively modulate the kinase activity of *SRK3* (Cabrillac *et al.* 2001).

SI recognition genes identified from anther

In *Brassica* species, pollen coat proteins (PCPs), which are derived from anther tapetum, are essential for pollen development. Analysis of PCPs, successfully lead to identification of the male *S* determinant (Heslop-Harrison 1975). Initially, *SLG*-interactive PCPs were searched (Doughty *et al.* 1993), and these interactive molecules were then characterized as cysteine-rich small proteins (Hiscock *et al.* 1995; Stanchev *et al.* 1996; Toriyama *et al.* 1998; Doughty *et al.* 1998; Takayama *et al.* 2000a). The cysteine-rich small proteins were shown to be a < 10kDa basic pollen coat proteins (PCP) by an elegant in vitro bioassay in which PCPs were isolated and fractionated (Stephenson *et al.* 1997). Interestingly, a gene encoding PCP-like protein, *SP11*, was found to be located at the *S*-locus, near *SRK* in *B. rapa*; thus *SP11* was the most likely candidate for the male *S* determinant (Suzuki *et al.* 1999). Subsequently, it was demonstrated that *SP11* is the male *S* determinant by using bioassay and transgenic experiments in *B. rapa* (Takayama *et al.* 2000b; Shiba *et al.* 2001). Furthermore, recently we showed the self-compatible phenotype in a

transgenic line of *B. rapa* after silencing of *SP11* gene (Jung *et al.* 2012). Around the same time, a gene termed *SCR* (*S*-locus cysteine-rich protein) in *B. oleracea* was also cloned, and identified as the male *S* determinant (Schopfer *et al.* 1999). SCRs represent a new class of small, secreted, cysteine-rich proteins, distinguishable from members of the PCP family. The *SCR* gene is tightly linked to the *SLG*/*SRK* pair and shows an anther specific developmentally regulated expression profile. Comparisons of the deduced amino acid sequence of SCR proteins from 21 *S* haplotypes revealed a high degree of polymorphism (Watanabe *et al.* 2000). Different studies also showed that *SP11* and *SCR* are identical (Suzuki *et al.* 1999; Takayama *et al.* 2000b; Schopfer *et al.* 1999). On the other hand, for studying the function of *SP11* in SI signaling cascade, *Arabidopsis* species and *B. rapa* were used because their whole genome sequence have been determined and other genetic tools (e.g. T-DNA tag lines, many ecotypes and microarray expression data) are available. Tsuchimatsu, Suwabe and co-workers introduced *SP11* into the Wei-1 ecotype of *A. thaliana*, which has a functional *SRK* but shows SC, to produce a self-incompatible *A. thaliana* (Tsuchimatsu *et al.* 2010). Furthermore, we developed self-compatible *B. rapa* after silencing *SP11* gene in a self-incompatible *B. rapa* line (Fig. 1; Jung *et al.* 2012). After identification of *SLG*, *SRK* and *SP11* genes, many allelic genes were isolated (Watanabe and Hinata 1999; Watanabe *et al.* 2008), which made it possible for us to calculate the allelic diversity.

***S*-linked genes involved in SI recognition**

To date, several *S*-linked genes have been identified in the region downstream of the *SLG* genes (~15kb). Boyes and Nasrallah (1995) have described a gene, designated *SLA* (*S*-locus anther), of *B. oleracea* with anther-specific expression but Pastuglia *et al.* (1997b) showed that a functional *SLA* gene is not required for the SI response in *Brassica*. Two *S*-linked genes *SLL1* and *SLL2* (for *S* locus-linked genes 1 and 2, respectively) of *B. napus* are located downstream of *SLG* gene (Yu *et al.* 1996). Although *SLL1* and *SLL2* are linked to the *S*-locus region, it is not clear whether these genes function in self-incompatibility or serve some other cellular roles in pollen-pistil functions. The *S*-linked ClpP (protease homologue) gene also seems unlikely to function in the SI reaction because of its expression pattern (Letham and Nasrallah 1998). Alongside, the genes that exhibit sequence similarity to the *SLG* genes, but are genetically unlinked to the *S*-locus, are designated *S*-locus-related (*SLR*) genes. Three members of this family, denoted *SLR1*, *SLR2*, and *SLR3*, have been shown to be expressed (Lalonde *et al.* 1989; Boyes *et al.* 1991; Cock *et al.* 1995). Genes *SLR1* and *SLR2* are linked to each other but unlinked (like *SLR3*) to *S*-locus thus they do not participate directly in the recognition of self and non-self in self-incompatibility. Suzuki *et al.* (1999) have demonstrated that four of the new *SP* genes (*S*-locus protein *SP5*, *SP6*, *SP8* and *SP11*) located at *S*-locus of the *S*⁹ haplotype appear to be expressed

Fig. 1. Schematic representations of the *S*⁶⁰-SP11RNAi vector construct and consequent pollen-tube behaviors of non-transgenic and RNAi transgenic plants (Jung *et al.* 2012).

specifically in reproductive organs. An interesting finding in the study of Suzuki *et al.* (1999) is that predicted mature protein of *SP11* gene is the same protein as male determinant (pollen ligand) of the SI. Schopfer *et al.* (1999) have published this protein, which they named *S*-locus cysteine-rich protein (SCR), soon after the publication of Suzuki *et al.* (1999).

Mechanisms involved in the SI reaction

To date, three polymorphic SI genes have been identified at the *S*-locus of *Brassica* species. The *SRK* gene and *SP11* gene determine the *S* specificity of the stigma (Takasaki *et al.* 2000) and the pollen (Schopfer *et al.* 1999; Shiba *et al.* 2001; Shiba *et al.* 2002; Suzuki *et al.* 1999; Takayama *et al.* 2000), respectively. The *SLG* enhances the SI recognition reaction (Takasaki *et al.* 2000), perhaps by stabilizing *SRK* (Dixit *et al.* 2000). The physical interaction between *SP11* and *SRK*, and the *SRK*-related signaling cascade following this interaction were the next research targets after identification of both male and female *S* determinants. Recent biochemical analysis has suggested that *SP11* functions as a sole ligand to activate its cognate *SRK* specifically (Takayama *et al.* 2001). In the case of self-pollination, SCR protein (pollen ligand) is bound to *SRK*, resulting in activation of the receptor and initiation of a signal transduction cascade that ultimately leads to pollen inhibition (Kao and McCubbin 2000). It is possible that an *SLG* has a role in the binding of its cognate *SRK* with the pollen ligand by forming a complex with the *S*-domain of the *SRK* and facilitating the process of the recognition reaction (Takasaki *et al.* 2000).

In the *Brassica* stigmatic papillae, *SRK* has been found to be localized to endosomes and the plasma membrane where it is positioned to perceive and bind to the SCR/SP11 protein present in the pollen coat of the self-incompatible pollen (Kachroo *et al.* 2001; Takayama *et al.* 2001; Ivanov and Gaude 2009). Interestingly, in the absence of the SCR/SP11 ligand, *SRK* forms ligand independent dimers (Giranton *et al.* 2000; Shimosato *et al.* 2007). The extracellular region contains hypervariable subdomains for ligand-specificity as well and presents a high affinity binding site at the plasma membrane for haplotype-specific SCR/SP11 ligand binding (Kemp and Doughty 2007;

Shimosato *et al.* 2007; Boggs *et al.* 2009). Binding of the SCR/SP11 ligand results in the phosphorylation and activation of the *SRK* kinase domain, which in turn leads to further signaling within the stigmatic papilla to produce the pollen rejection response (Kachroo *et al.* 2001; Takayama *et al.* 2001; Shimosato *et al.* 2007). Shimosato *et al.* (2007) showed that both the *S*-domain and membrane-anchoring domain were necessary for allele-specific interaction by using crosslinking and immunological methods. In contrast, Kachroo *et al.* (2001) showed that only the *S*-domain of *SRK* could interact with SP11 in the allele-specific manner by using an immunoprecipitation method. Little is understood about the molecular mechanisms in the *SRK*-mediated signal transduction pathway. In the future, after determination of the tertiary structure of *SRK*, this inconsistency is likely to be resolved.

In order to clarify the *SRK* signaling cascade, several *SRK*-interacting molecules were identified and characterized (Gu *et al.* 1998; Stone *et al.* 1999, 2003; Cabrillac *et al.* 2001; Vanoosthuysen *et al.* 2003; Murase *et al.* 2004; Kakita *et al.* 2007b). Receptor kinase activation typically results in downstream signaling proteins interacting with the activated kinase domain, and consistent with this, several proteins have been found to interact with the *SRK* kinase domain (Bower *et al.* 1996; Gu *et al.* 1998; Vanoosthuysen *et al.* 2003; Kakita *et al.* 2007a, b). Two *Brassica* thioredoxin *h* proteins, *THL1* and *THL2*, were isolated first (Bower *et al.* 1996). The thioredoxin *h* proteins were found to negatively regulate *SRK* as well as the self-incompatibility response and were proposed to maintain *SRK* in an inactive state in unpollinated stigmas (Cabrillac *et al.* 2001; Haffani *et al.* 2004). Interestingly, *THL1* was found to partially co-localize with *SRK* in the endosomes, but could not be detected at the plasma membrane (Ivanov and Gaude 2009); thus, whether thioredoxin *h* inhibition of *SRK* occurs at the plasma membrane is not clear. Nevertheless, this inhibition is proposed to be related with SCR/SP11 ligand binding. A recessive *mod* mutation which causes a breakdown in *Brassica* self-incompatibility led to the discovery of the *M*-Locus Protein Kinase (MLPK) encoded by the *mod* locus (Murase *et al.* 2004). MLPK was found to have serine/threonine activity, and membrane localization of MLPK is required for the self-incompatibility response

(Murase *et al.* 2004; Kakita *et al.* 2007a). MLPK is predicted to interact with SRK at the plasma membrane, and this interaction was demonstrated in BY-2 cells as well as in phosphorylation related studies (Kakita *et al.* 2007a, b). Thus, MLPK and SRK may form a complex to activate downstream signaling proteins to set the self-incompatibility cellular cascade in motion.

An example of a downstream player is another *Brassica* interacting protein, ARC1, an E3 ubiquitin ligase also required for the self-incompatibility response (Gu *et al.* 1998; Stone *et al.* 1999). The ARC1 protein contains a novel N-terminal domain (UND), followed by a U-box domain and an ARM repeat domain (Samuel *et al.* 2006). The binding of ARC1 to the phosphorylated SRK kinase domain *in vitro* is mediated by the ARM repeat domain (Gu *et al.* 1998), but interestingly, ARC1 serves as a much better substrate for *in vitro* phosphorylation by MLPK, supporting the idea of an SRK-MLPK complex activating ARC1 (Samuel *et al.* 2008a). Because ARC1 is proposed to target a substrate for degradation in the self-incompatibility response, it is reasonable to speculate that ARC1 functions as an inhibitor of compatibility factors in pollination events as a pollen rejection mechanism (Stone *et al.* 2003). Not as

much is known about the cellular events occurring in a stigmatic papilla during a compatible pollen interaction. Whether activation of this ARC1 E3 ubiquitin degradation pathway is directly responsible for the rejection of self-incompatible pollen has yet to be confirmed, but recently, a new compatibility factor, Exo70A1, which may be the substrate for ARC1's ubiquitin-mediated degradation pathway in this self-incompatibility response, was identified in both *Brassica* and *Arabidopsis* (Samuel *et al.* 2009). A schematic model of the current understanding of *Brassica* SI reaction is shown in Fig. 2.

Dominance relationships between *S* alleles

The majority of the members of the Brassicaceae plant genus *Brassica* possess a strong sporophytic SI system. An important characteristic of *Brassica* SI is the dominance relationships between *S*-alleles, which are a consequence of the sporophytic behavior of *S* genes (Thompson and Taylor 1966, Hatakeyama *et al.* 1998, Bateman 1952, 1954, 1955). Thus, the SI phenotype of pollen as well as stigma is determined by relationships between the two *S*-haplotypes carried by its parent (Bateman 1955). In other words, a codominant or a dominant/recessive relationship

Fig. 2. Schematic model for self-pollen recognition in *Brassica* species. Male and female *S* determinant genes, *SP11* and *SRK*, 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. During self-pollination, *SP11* molecules penetrate into the papilla cell wall, and interact with *SRK* in an *S*-allele-specific manner. Phosphorylated *SRK* interacts with MLPK. After the subsequent signal transduction, which has not yet been determined, rejection of the self-pollen occurs (Watanabe *et al.* 2012).

between the two *S*-haplotypes influences the ultimate SI phenotype of both pollen and stigma (Thompson and Taylor 1966). The following observations have been made about dominance relationships among *S*-haplotypes: (1) co-dominance is more frequent than dominance/recessiveness; (2) dominance/ recessiveness in the pollen is observed more frequently than that in the stigma; (3) dominance relationships among stigmas are different from those among pollen; and (4) dominance relationships are non-linear, and are more frequent in the stigma than in the pollen (Thompson and Taylor 1966; Ockendon 1975; Visser *et al.* 1982; Hatakeyama *et al.* 1998). Discovery of the molecular mechanisms of dominance relationships has been based on these genetic features.

The different mechanisms operating in the male and female side in the dominance relationships of SI are

consistent with the four genetic characteristics described above. Dominance relationships of SI at the female side appear to be post-transcriptionally regulated by *SRK*, unlike transcriptional suppression of *SP11* (Hatakeyama *et al.* 2001). They investigated whether the stigmatic *S*-determinant of *SRK* was involved in determining the dominance relationships of stigma using five *S*-homozygotes carrying an *SRK28* transgene. They showed that the dominance relationship between the *SRK28* transgene and each of the endogenous *S*-haplotypes was identical to that between the *S*²⁸-haplotype and the respective endogenous *S*-haplotype. Moreover, in the *S*⁴³*S*⁴³-homozygote carrying the *SRK28* transgene, in which the *S*⁴³ phenotype in the stigma was masked by the presence of *SRK28*, the transcript level of *SRK28* was found to be much lower than that of *SRK43*. These results suggest that the dominant/

Fig. 3. Schematic model for molecular mechanisms of dominance relationships at the pollen side. (A) In the case where *S*¹ is dominant over *S*², dominant transcripts of *SP11*, *S*¹-*SP11*, are specifically expressed in the *S*¹*S*² heterozygote. However, *S*²-*SP11* transcripts are not detected in the *S*¹*S*² heterozygote on RNA gel blot analysis. The results demonstrate that the dominance relationship at the pollen side is regulated at the transcriptional level. (B) In the dominant *S*-allele, small RNA, termed Smi (*SP11* methylation inducer), is specifically produced, and its nucleotide sequence is highly similar to the promoter region of the recessive *SP11* gene. This small RNA induces the methylation of recessive *SP11*, and represses the recessive *SP11* at the transcriptional level (Watanabe *et al.* 2012).

recessive relationships between *S*-haplotypes in the stigma are determined by SRK itself, but not as a result of its relative transcription level.

Regarding dominance relationships in pollen, the pollen determinant *SP11* from pollen-recessive *S*-haplotypes has not been identified, and how the relationships are determined is not known. The dominance relationships of SI are regulated by transcription of *SP11* in Brassica pollen. In sporophytic tapetum cells of anthers of *S* heterozygous plants, *SP11* derived from the dominant allele is normally expressed, whereas expression of the recessive *SP11* is significantly suppressed (Fig. 3A) (Shiba *et al.* 2002; Kakizaki *et al.* 2003). Interestingly, linear dominance relationships ($S^9 > S^{44} > S^{60} > S^{40} > S^{29}$) are also observed in *B. rapa* (Hatakeyama *et al.* 1998; Kakizaki *et al.* 2003). In this case, dominance/recessiveness of S^{44} , S^{60} , S^{40} alleles could be altered, indicating involvement of epigenetic regulation (Kakizaki *et al.* 2003). From observation of the methylation level of the promoter region of *SP11* in several *S* heterozygotes, it was shown that the recessive *SP11* is specifically methylated in *S* heterozygotes (Shiba *et al.* 2006). Recently, it was further demonstrated that the small RNA produced from the dominant allele could activate methylation of the recessive allele. In transgenic experiments, S^{60} (class II) transformants with a class-I-derived small RNA region (S^9) showed SC, and their promoter regions were highly methylated, as in the S^9S^{60} heterozygote, indicating that the small RNA from the dominant allele functions in trans to induce transcriptional silencing of the recessive allele (Fig. 3B; Tarutani *et al.* 2010). However, it has been suggested that pollen-recessive *S*-haplotypes have a set of *SLG* and *SRK* called class II, whereas pollen-dominant *S*-haplotypes have a different set called class I (Nasrallah and Nasrallah 1993).

Furthermore, based on the sequence diversity of the SI genes, *S* haplotypes are classified into class I and class II; the amino acid sequence similarity of SLG and SRK is about 65% between classes and 80–90% within classes (Watanabe *et al.* 2001). On the other hand, amino acid sequence identities among class II SP11s are 62.3 to 94.6%, rather higher compared with those of class I SP11s which ranges from 19.5 to 76.1% (Shiba *et al.* 2002). Interestingly, class-I *S* haplotypes are dominant over class-II ones in the

dominance relationships on the pollen side (Hatakeyama *et al.* 1998, Nasrallah and Nasrallah 1993, Thompson and Taylor 1966). To date, class II *SLGs* have been found only in pollen-recessive *S*-haplotypes, which include S^{29} -, S^{40} -, S^{44} -, and S^{60} -haplotypes of *B. rapa* (syn. *campestris*) (Hatakeyama *et al.* 1998; Takasaki *et al.* 2000) and $S2$ -, $S5$ -, and $S15$ -haplotypes of *B. oleracea* (Chen and Nasrallah 1990; Scutt and Croy 1992; Cabrilla *et al.* 1999). The pairwise sequence identity among class II *SLGs* is in excess of 86%, whereas that between class I and class II *SLGs* is only ~60 to 70%. The extracellular domains of pollen-dominant and pollen-recessive SRKs also can be placed into these two classes in a similar manner. These analytical results on stigmatic *S*-locus proteins suggest that class II *S*-haplotypes have an origin quite different from that of class I *S*-haplotypes (Uyenoyama 1995; Kusaba *et al.* 1997; Schierup *et al.* 2001).

Future outlook

SI is one of the most interesting phenomena in sexual reproduction and the molecular genetics processes in sexual plant reproduction research have now been extensively dissected in Brassicaceae plants. In recent years, significant progress has been made in strengthening our understanding of the molecular and cellular steps behind the self-incompatibility response in the Brassicaceae. Such accumulation of biological and molecular genetic knowledge will also contribute to the understanding of interesting biological features, including cell-cell communication, ligand-receptor interaction, signal transduction, phosphorylation cascade, molecular evolution, allelic polymorphism and epigenetic regulation by small RNAs in the SI recognition reaction. However, there are areas that still require further dissection, and current research is aimed at more fully understanding the specific signaling events that take place in the stigmatic papillae following pollination. For example, efforts have focused on identifying residues within the SRK extracellular domain that are essential for its activation by the SCR/SP11 ligand. It appears that a surprisingly small number of residues are required, and that the three-dimensional conformation of these regions is a determining factor (Boggs *et al.* 2009). Outstanding questions also encompass the regulatory role of *MLPK*, in relation to *SRK*, on downstream signaling

steps including *ARCI* and perhaps other unknown signaling proteins or events. To elucidate a complete overview of the peptide signaling, further analysis of protein-protein interaction by the use of yeast two-hybrid system, protein cross-linking, etc. is required (Hattori *et al.* 2009; Shinya *et al.* 2010), in addition to the genetic analysis described above. Furthermore, when the tertiary structure of *SRK* has been carried out and established, the precise *SP11-SRK* allelic-specific interaction will provide new insight into *SP11*-induced *SRK* activation. In summary, the dissection of cellular events activated in the self-incompatibility response and understanding molecular mechanism of the SI recognition reaction in the Brassicaceae has started to uncover which will also be important in establishing F₁ hybrid seed production in the future.

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