Comparison of the Strength of Self-incompatibility between *S* Haplotypes in Chinese Cabbage

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Summary

The strength of self-incompatibility is an important factor for F_1 seed production to avoid inbreeding seed contamination in cruciferous vegetables. Cruciferous self-incompatibility is sporophytically controlled by a single genetic locus (*S* locus) with a series of alleles and has a dominance relationship among *S* haplotypes. However, the association between the strength of self-incompatibility and dominance relationship has not been elucidated. In this study, we generated *S* homozygous plants derived from commercial F_1 hybrid cultivars of *Brassica rapa* var. *pekinensis* L. (Chinese cabbage), and compared the strength of self-incompatibility with three independent *S* haplotype combinations. Pollination tests revealed that there was no difference of the strength of self-incompatibility between two class-I *S* haplotype combinations either with or without dominance relationship. However, self-incompatibility in class-II *S* homozygotes, which is recessive in the pollen, was weaker than that in class-I *S* homozygotes. These results indicate that the strength of self-incompatibility between *S* haplotypes is independent from dominance relationship.

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Key words : *Brassica rapa,* Dominance relationship, Self-incompatibility, *S* haplotype

Many species in the genus *Brassica* have a self-incompatibility system, which is controlled by a single *S* locus with multiple alleles (Bateman, 1955). The determinants of self-recognition specificity in the stigma and the pollen have been identified. The female determinant is a membrane spanning serine threonine kinase, named SRK (*S* receptor kinase) (Stein *et al*., 1991, Takasaki *et al*., 2000), which has an extracellular domain (*S* domain), a transmembrane domain, and an intracellular domain (kinase domain). The male determinant is a small cysteine rich protein, named SP11/ SCR (*S* locus protein 11/ *S* locus cysteine rich) (SP11 hereafter) (Schopfer *et al*., 1999, Suzuki *et al*., 1999). SP11 binds SRK in an allele-specific manner (Kachroo *et al*., 2001, Takayama *et al*., 2001), and it is generally thought that this SP11-SRK binding induces reactions that lead to self-pollen rejection. The process leading to pollen tube rejection from *S*-haplotype-specific interaction is not well understood, but some genes participating in the self-incompatibility reaction after interaction of SRK and *SP11* have been identified (Fujimoto and Nishio, 2007). One of the genes is *MLPK (M* locus protein kinase), which was isolated as a candidate gene of *M* by map-based cloning (Murase *et al*., 2004). The *M* locus controlling self-compatibility of 'Yellow Sarson' is independent of the *S* locus and epistatic to the *S*-locus gene (Hinata *et al*., 1983). MLPK of 'Yellow Sarson' has one amino acid substitution caused by a single nucleotide change that leads to the loss of autophosphorylation activity (Murase *et al*., 2004). MLPK belongs to a subfamily of receptor-like cytoplasmic kinase (RLPK) and interacts directly with SRK to transduce self-incompatibility reaction (Kakita *et al*., 2007).

SRK and *SP11* are closely linked to each other in the *S* locus, and the alleles of these two genes are transmitted to progeny as one set. Therefore, a set of alleles of *SRK* and *SP11* is termed *S* haplotype (Nasrallah and Nasrallah, 1993). About 50 and 30 *S* haplotypes have been identified in *Brassica oleracea* and *Brassica rapa,* respectively (Ockendon, 2000, Nou *et al*., 1993). Nucleotide sequences of the first exon of *SRK* encoding the *S* domain and *SP11* of many *S* haplotypes have been determined in *B. rapa, B. oleracea,* and *Raphanus sativus* (Watanabe *et al*., 2000, Sato *et al*., 2002, Okamoto *et al*., 2004, Takuno *et al*., 2007). Based on nucleotide sequences of the *S* locus genes, *S* haplotypes are classified into two groups, class-I and class-II (Nasrallah *et al*., 1991).

Because self-incompatibility in *Brassica* is sporophytically controlled, there are dominance relationships of *S* haplotypes in the stigma and the pollen (Thompson and Taylor, 1966). The dominance relationships are different between the stigma and the pollen (Thompson and Taylor 1966, Hatakeyama *et al*., 1998), and co-dominance is observed more frequently in the stigma than in the pollen. The dominance relationship in the stigma is considered to be determined by the SRK protein itself (Hatakeyama *et al*., 2001). In pollen, class-I *S* haplotypes are generally dominant over class-II *S* haplotypes (Nasrallah *et al*., 1991), and dominance relationship in pollen is controlled at the mRNA level (Kusaba *et al*., 2002,

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Shiba *et al*., 2002). This transcriptional suppression of recessive *SP11* has been reported to be controlled by *de novo* DNA methylation of the promoter region of recessive *SP11* through small RNAs, which is expressed from *S* locus of dominant allele (Shiba *et al*., 2006, Tarutani *et al*., 2010).

Many cultivars of Brassicaceae vegetables, e.g., cabbage, broccoli, cauliflower, Chinese cabbage, turnip, and radish, are F_1 hybrids and their seeds are produced using the self-incompatibility system or cytoplasmic male sterility. As for the yield of F_1 hybrid seeds, F_1 hybrid breeding using the self-incompatibility system is much superior to using cytoplasmic male sterility. However, instability of self-incompatibility influenced by environmental factors such as high temperature sometimes results in production of low-quality seeds containing a high proportion of selfed seeds. Strong and stable self-incompatibility is required for F_1 hybrid breeding. Although no *S* haplotypes providing strong self-incompatibility are known, class-II *S* haplotypes are known to exhibit a weak self-incompatibility phenotype (Nasrallah and Nasrallah, 1993).

In this study, we compared the strength of self-incompatibility between two *S* homozygous lines segregated from F_2 population, which is derived from commercial F_1 hybrid cultivars, with three independent *S* haplotype combinations. There is no difference of strength of self-incompatibility between two class-I *S* haplotypes with or without dominance relationship, but self-incompatibility in class-II *S* homozygous plants was weaker than that in class-I *S* homozygous plants.

MATERIALS ANS METHODS

Plant materials

F_1 hybrid cultivars of Chinese cabbage, *B. rapa*, 'CR-Seiga 65' (*S-46/54*) (Ishii, Shizuoka, Japan), 'Kien 75' (*S-54/S-60*) (Nihon-Norin, Tokyo, Japan), and 'CR-Ryutoku' (*S-54/S-99*) (Watanabe, Miyagi, Japan), and selfed progenies (F_2) derived from these three F_1 hybrid cultivars were used as materials. F_2 plants were obtained by bud pollination. The *S* haplotypes of these three F_1 cultivars has been determined (Sakamoto *et al*., 2001). *S-46, S-54*, and *S-99* are class-I *S* haplotypes, and *S-60* is class-II *S* haplotypes (Sato *et al*., 2002, Takuno *et al*., 2007).

Determination of *S* haplotypes

S haplotypes of F_2 plants were identified by PCR or PCR-RFLP analysis of *SLG* (*S* locus glycoprotein), which is linked to *S* locus, using primer pairs PS5/15 and PS3/21 (Nishio *et al*., 1996). PS5 (5'- ATGAAAGGCGTAAGAAAAACCTA-3') and PS15 (5'- CCGTGTTTTATTTTAAGAGAAAGAGCT-3') are specific for the class-I *SLG*. PS3 (5'-ATGAAAGGGGTACAGAACAT-3') and PS21 (5'-CTCAAGTCCCACTGCTGCGG-3') are specific for the class-II *SLG*. Genomic DNAs of these plants were isolated from leaves using a modified NaI method according to Sakamoto *et al*., 2000.

Pollination test

Pollination tests were performed using more than three flowers with three replications. After anthesis, the stigma was covered with a layer of pollen grains, and the pollinated flowers were kept at 20℃ for one day. Pollen tubes in the stigma were observed under a UV fluorescence microscope after staining with aniline blue (0.1% aniline blue in 0.1M K_3PO_4). The level of self- and cross-incompatibility was scored as follows: --, no pollen tube penetrating a papilla cell; -, less than 5 pollen tubes penetrating papilla cells; +-, more than 6 and less than 20 pollen tubes penetrating papilla cells; +, more than 21 pollen tubes penetrating papilla cells. The numbers of pollen tubes penetrating papilla cells evaluated the strength of self-incompatibility.

RESULTS

To examine whether the strength of self-incompatibility results in *S* haplotypes, mean values of about ten plants of pollen tube numbers penetrating papilla cells in stigma (PTN) were compared between two *S* homozygotes segregated from an F_2 population derived from same F_1 cultivars, which are *S* heterozygotes. The genetic background of each individual is random in F_2 generation, but comparing the average of PTNs minimizes the background effect. The three F_1 cultivars, 'CR-Seiga 65' (*S-46/S-54*), 'Kien 75' (*S-54/S-60*), and 'CR-Ryutoku' (*S-54/S-99*) were used for obtaining the two types of *S* homozygotes in F_2 generations to compare the strength of self-incompatibility between *S* haplotypes.

At first, we examined the dominance relationship in both pollen and stigma to investigate the possibility that dominant or recessive *S* haplotype may associate with weak or strong self-incompatibility. The stigmas of *S-46/S-54* were incompatible with the pollen grains of *S-46* homozygote,

Table 1 Dominance relationship in both pollen and stigma

Stigma	Pollen		
	S-46/S-46	*S-54/S-54*	*S-46/S-54*
S-46/S-46	--	+	--
S-54/S-54	+	--	+
S-46/S-54	--	+	--
	S-54/S-54	*S-60/S-60*	*S-54/S-60*
S-54/S-54	--	+	--
S-60/S-60	+	--	+
S-54/S-60	--	--	--
	S-54/S-54	*S-99/S-99*	*S-54/S-99*
S-54/S-54	--	+	--
S-99/S-99	+	--	--
S-54/S-99	--	--	--

PTN = 0, --; PTN = 1～5, -; PTN = 6～20, -+; PTN ＞20, +

Pollination tests were performed using more than three flowers with three replications, and the most frequent score in each cross combination was represented.

Table 2 The pollination test of selfed progenies of 'CR-Seiga 65'

S-46/S-46	1st	2nd	3rd	*S-54/S-54*	1st	2nd	3rd
1	--,--,--	--,--,--	--,--,--	1	-,--,--	--,--,--	--,--,--
2	--,--,--	--,--,--	--,--,--	2	--,--,--	--,--,--	--,--,-
3	--,--,--	--,--,--	--,--,--	3	--,--,--	--,--,--	--,--,--
4	--,-,--	--,--,--	--,--,--	4	--,--,--	-,--,--	--,--,--
5	--,--,--	--,--,--	--,--,--	5	--,--,--	--,--,--	--,--,--
6	--,--,--	--,-,--	--,--,--	6	-,-,--	--,-,--	--,--,--
7	--,-,--	-,--,--	--,--,--	7	-,--,--	--,--,--	--,--,--
8	-,--,--	--,--,--	-,-,-	8	--,--,--	-,-,--	--,--,--
9	-,-,-	--,--,--	--,--,--	9	--,--,--	-,--,--	--,--,--
10	-,-,--	--,--,--	--,-,--				

PTN = 0, --; PTN = 1～5, -; PTN = 6～20, -+; PTN > 20, +

Table 3 The pollination test of selfed progenies of 'Kien 75'

S-54/S-54				*S-60/S-60*			
1	--,--,--	--,--,--	--,--,--	1	--,--,--	--,--,--	--,--,--
2	--,--,--	--,--,--	--,--,--	2	--,--,-	--,--,--	--,--,--
3	--,--,--	--,--,--	--,--,--	3	--,--,--	--,--,--	-,-,--
4	--,--,--	--,--,--	--,--,--	4	--,-,--	--,--,--	--,--,--
5	-,-,--	--,--,--	--,--,--	5	+,+,+	--,--,+	+,+,-
6	--,--,--	--,--,--	--,--,--	6	-,--,--	--,--,--	--,--,--
7	--,--,--	--,--,--	--,--,--	7	--,--,--	--,--,--	--,--,--
8	--,--,--	--,--,--	--,--,--	8	--,-,--	--,--,--	--,--,-
9	--,-,--	--,--,--	--,--,--	9	-,--,--	--,--,--	--,--,--
10	--,--,--	--,-,--	--,--,--	10	--,--,--	--,--,--	--,-,-
11	--,--,--	--,--,--	--,--,--	11	-,--,--	--,--,--	--,-,--
12	--,--,--	--,--,--	--,--,--	12	--,--,-	-,-,--	-,--,--
13	--,--,--	--,--,--	--,--,--	13	--,-,--	--,--,--	--,-,-
14	--,--,--	--,--,--	--,--,--	14	--,--,--	--,--,--	--,--,--
15	--,--,--	--,--,--	--,--,--	15	--,--,--	--,--,--	--,--,--
				16	-+,-,-	-,--,-	-,-,-
				17	-,--,--	--,--,--	--,--,-

PTN = 0, --; PTN = 1～5, -; PTN = 6～20, -+; PTN > 20, +

Table 4 The pollination test of selfed progenies of 'CR-Ryutoku'

S-54/S-54				*S-99/S-99*			
1	--,--,--	--,--,--	--,-,--	1	--,--,--	--,--,--	--,--,--
2	--,--,--	--,--,--	--,--,--	2	--,--,--	--,--,--	--,--,--
3	--,--,--	--,--,--	--,--,--	3	--,--,--	--,-,--	--,--,--
4	--,--,--	--,--,--	--,--,--	4	--,--,--	--,--,--	--,--,--
5	--,--,--	--,--,--	--,--,--	5	--,--,--	--,--,--	--,--,--
6	--,--,--	--,--,-	--,--,-	6	--,--,--	--,--,--	-,--,--
7	--,--,--	--,--,--	--,--,--	7	--,--,--	--,--,--	--,--,--
8	--,--,--	--,--,--	--,-,--	8	--,--,--	--,--,--	--,--,--
9	-,--,--	--,--,--	--,--,--	9	--,--,--	--,--,--	--,--,--
				10	--,-,--	--,--,--	--,--,--
				11	--,--,--	--,--,--	--,--,--
				12	--,--,--	--,--,--	--,--,--
				13	--,--,--	--,--,--	--,--,--
				14	--,--,--	--,--,--	--,--,--
				15	-,--,--	--,--,--	--,--,--

PTN = 0, --; PTN = 1～5, -; PTN = 6～20, -+; PTN > 20, +

while compatible with the pollen grains of *S-54* homozygote (Table 1). The pollen grains of *S-46/S-54* were incompatible with the stigma of *S-46* homozygote, while compatible with the stigma of *S-54* (Table 1). These results indicated that *S-46* is dominant over the *S-54* in both pollen and stigma. The stigmas of *S-54/S-60* were incompatible with the pollen grains of *S-54* or *S-60* homozygotes (Table 1). The pollen grains of *S-54/S-60* were incompatible with the stigma of *S-54* homozygote, while compatible with the stigma of *S-60* (Table 1). These results indicate that *S-54* is dominant over the *S-60* in pollen, while *S-54* and *S-60* were co-dominant in the stigma. The stigmas of *S-54/S-99* were incompatible with the pollen grains of *S-54* or *S-99* homozygotes (Table 1). The pollen grains of *S-54/S-99* were incompatible with the stigma of *S-54* or *S-99* homozygotes (Table 1). These results indicate that *S-54* and *S-99* were co-dominant both in stigma and pollen.

S haplotypes of 90 plants in the selfed progenies of 'CR-Seiga 65' were analyzed by PCR-RFLP analysis of *SLG*. Eighteen and twenty-one plants were *S-46* and *S-54* homozygotes, respectively, and 51 were heterozygotes of *S-46/S-54*. The PTN was examined after self-pollination in ten *S-46* and nine *S-54* homozygotes (Table 2). The mean values of PTNs in *S-46* and *S-54* were 0.19 ± 0.08 and 0.35 ±

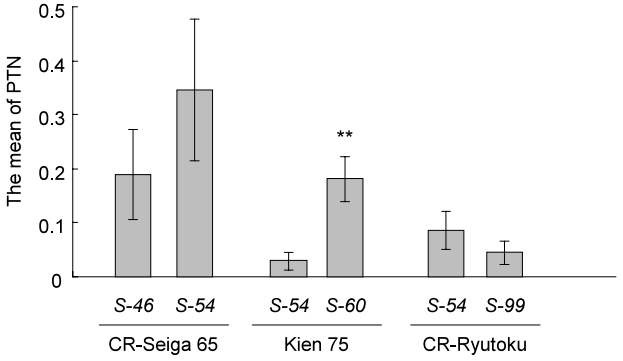

Fig. 1 Comparison of the strength of self-incompatibility between two S haplotypes. The numbers of pollen tubes penetrating papilla cells (PTN) was used for evaluation of the strength of self-incompatibility. Histogram shows means values ± s.d. obtained from each pollination test. **, $p<0.01$.

0.13, respectively (Fig. 1), and there is no significant difference between them.

S haplotypes of 77 plants in the selfed progenies of 'Kien 75' were analyzed by PCR using class-I and class-II *SLG* specific primer sets. Sixteen and twenty plants were *S-54* and *S-60* homozygotes, respectively, and 41 were heterozygotes of *S-54/S-60*. The mean values of PTNs were examined after self-pollination in fifteen *S-54* and seventeen *S-60* homozygotes. As one line (No. 5) of *S-60* homozygotes showed weak self-incompatibility (Table 3), we removed this line to calculate the mean value of PTNs. The mean values of PTNs in *S-54* and *S-60* (except for No. 5) were 0.030 ± 0.002 and 0.18 ± 0.04, respectively (Fig. 1), and PTN in *S-60* is significantly higher than that in *S-54* ($p<0.01$), indicating that the strength of self-incompatibility in *S-60* is weaker than that in *S-54*.

S haplotypes of 55 plants in the selfed progenies of 'CR-Ryutoku' were analyzed by PCR-RFLP analysis of *SLG*. Eleven and sixteen plants were *S-54* and *S-99* homozygotes, respectively, and 28 were heterozygotes of *S-54/S-99*. The PTN was examined after self-pollination in nine *S-54* and fifteen *S-99* homozygotes (Table 4). PTNs in *S-54* and *S-99* were 0.086 ± 0.04 and 0.044 ± 0.02, respectively (Fig. 1), and there is no significant difference between them.

DISCUSSION

In this study, we compared the strength of self-incompatibility between two *S* haplotypes with three combinations. Between *S-46* and *S-54* homozygotes, there was dominance relationship both in pollen and stigmas, and no difference in strength of self-incompatibility. By contrast, between *S-54* and *S-60* homozygotes, there was dominance relationship in pollen, and self-incompatibility in *S-60* homozygotes was weaker than that in *S-54* homozygotes. These results indicate that the strength of self-incompatibility between *S* haplotypes is independent from dominance relationship.

Although no *S* haplotypes providing strong self-incompatibility are known, class-II *S* haplotypes are known to exhibit a weak self-incompatibility phenotype (Nasrallah and Nasrallah, 1993). Among class-II *S* haplotypes, *S-15* in *B. oleracea* has been found to show the weakest incompatibility, perhaps due to the low affinity between SP11 and SRK, but *S-60* in *B. rapa* did not show weak self-incompatibility (Sato *et al*., 2006). Though *S-60* homozygotes showed weaker self-incompatibility than *S-54* homozygotes, PTNs of *S-60* homozygotes (0.18 ± 0.04) did not show weak self-incompatibility. Moreover, PTNs of *S-60* were not larger than those of *S-54* homozygous lines derived from other F_1 hybrid cultivars, CR-Seiga 65 (PTNs = 0.35 ± 0.13). The F_1 hybrid cultivars in *B. rapa* and *B. oleracea* tended to have class-I/class-II *S* heterozygotes (Sakamoto *et al*., 2000, 2001). During the establishment of parental lines, strong self-incompatibility should be selected, suggesting that class-II *S* haplotypes may not lead to weak self-incompatibility. From these observations, we conclude that class-II *S* haplotypes do not always result in weak self-incompatibility.

It has been suggested that the strength of self-incompatibility is controlled by the genetic background (Ruffio-Chable *et al*., 1997), and the level of self-incompatibility is regulated by a single gene (Horisaki *et al*., 2003) or by multiple genes (Nasrallah and Wallace, 1968). Liu *et al*. (2007) have reported that the hypomorphic gene, *PUB8 (PLANT U-BOX 8),* influenced the strength of self-incompatibility by regulating expression of *S*-locus genes in *Arabidopsis thaliana*. Recently, five quantitative trait loci (QTL) controlling the level of self-incompatibility were identified using QTL mapping in *B. rapa,* and two of these QTL were localized with *SLG* on R07 and *MLPK* on R03 (Hatakeyama *et al*., 2010). Detection of QTL in *S* locus and in region with co-localized with *MLPK* suggests that the strength of self-incompatibility is influenced by interaction of *S* haplotypes and the genetic background. We found one weak self-incompatible line only from *S-60* homozygous lines. This weak self-incompatibility might be due to the interaction between *S-60* haplotype and the background. Self-incompatibility has become unstable due to several factors such as environmental factors, stage of flower, and genetic background (Ockendon, 1975), and self-fertilized seed contamination becomes a problem in F_1 hybrid seed production. In the process of F_1 hybrid breeding, the strong and stable self-incompatibility are desired. Further research will be required to identify factors related to the strength of self-incompatibility, which can be used for marker assisted selection.

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白菜のSハプロタイプ間における自家不和合性の強度の比較

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要　約

アブラナ科野菜の自家不和合性の強度は、F_1種子の生産において、自殖種子の混入を避けるために重要な要素である。アブラナ科植物の自家不和合性は、1つの複合遺伝子座（*S*複合座）によって胞子体型に制御されており、*S*ハプロタイプ間で優劣性が見られる。しかし、自家不和合性の強度と優劣性との関連性はあまり研究されていない。本研究では、*Brassica rapa*（白菜）のF_1品種に由来する*S*ホモ個体を作成し、3つの独立な*S*ハプロタイプの組合せについて自家不和合性の強度を比較した。優劣性の有無にかかわらず、2つのclass Ⅰの*S*ハプロタイプ間では自家不和合性の強度に違いが見られなかった。しかし、花粉において優劣性があるclass Ⅰとclass Ⅱの*S*ハプロタイプ間では、class Ⅱ*S*ハプロタイプの自家不和合性の強度はclass Ⅰ*S*ハプロタイプに比べて弱かった。これらの結果から、*S*ハプロタイプ間における自家不和合性の強度は優劣性とは独立していることが示唆された。

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