

A. Horisaki · N. Tanaka · S. Niikura

## The effectiveness of insect-pollination test to evaluate the level of self-incompatibility and their genetic analysis in radish (*Raphanus sativus* L.)

Received: 23 September 2002 / Accepted: 4 November 2002 / Published online: 5 April 2003  
© Springer-Verlag 2003

**Abstract** We have tried an insect-pollination self-incompatibility (SI) test to strictly evaluate the level of SI as a model for the actual F<sub>1</sub> seed production field using radish as experimental material. Twelve inbred lines, homozygous for the *S*-alleles, were used in the artificial self-pollination and the insect-pollination SI test. There was a positive correlation ( $r = 0.606$ ) between the results by these two methods. Some lines showed a low level of SI in the insect-pollination test despite showing a high level of SI in the artificial self-pollination test. On the other hand, no lines showing a low level of SI in artificial self-pollination had a high level of SI in insect-pollination. These results show that the insect-pollination SI test can be considered to be a more reliable and stricter method than the artificial self-pollination test with respect to an evaluation of SI levels. We have raised and analyzed an F<sub>2</sub> population and F<sub>3</sub> lines derived from an F<sub>1</sub> cross between a line showing a high level of SI (R00-04) and one showing a low level. The rate of self-seed settings of the F<sub>2</sub> population showed a binomial distribution. There were 39 high-level SI plants to 15 low-level SI plants. This result and F<sub>3</sub> progeny tests suggested that the high level of SI which R00-04 showed is controlled by a dominant gene.

**Keywords** Artificial self-pollination · Cruciferous plants · F<sub>1</sub> seed production · Self-incompatibility · *S*-gene

### Introduction

Self-incompatibility (SI) in the cruciferous plants is governed by a series of multiple alleles (*S*) acting sporophytically. That is, whether a pollen tube penetrates into the stigma or not depends on the *S*-genotype of the

sporophyte (Bateman 1952, 1955). However, there are genetic variations in SI levels (Ruffio-Chable et al. 1997), and SI can be overcome easily by many internal and external factors. Therefore, it is believed that SI is also regulated by genes other than the *S*-genes. A one-base deletion or an alternative transcript of the *SRK* gene located at the *S*-locus resulted in a low level of SI (Göring et al. 1993; Tantikanjana et al. 1993), and the *m* gene unlinked to the *S*-gene was related to the level of SI (Ikeda et al. 1997). In addition, estimates of genetic factors have been performed using the wild population in order to gain an understanding of the evolution of the plant breeding system (Good and Stephenson 2002). We have also reported the isolation of an *S*-locus gene, identified 37 *S*-alleles (*S*<sup>201</sup>–*S*<sup>237</sup>) using the inbred lines and landraces of radishes belonging to the cruciferous plants and have shown sporophytic SI (Niikura and Matsuura 1997, 1999, 2000). This so-called SI could be classified using the following characteristics: *S*-gene, level of SI and reaction level of SI to a 4% CO<sub>2</sub> gas treatment (Niikura and Matsuura 1999, 2000).

Most of the cruciferous vegetables are at present F<sub>1</sub> hybrid varieties, the seeds of which are produced using SI. However, SI is not actually a complete process. Therefore, stable F<sub>1</sub> seed production has been a hot topic for breeders for a long time. We have evaluated the level of SI using the rate of self-seed setting resulting from artificial self-pollinations to opened flowers. However, we have experienced that the F<sub>1</sub> purity, that is the ratio of F<sub>1</sub> hybrids in the harvested seeds, declined when using one particular parental line recognized as having a high level of SI. When a different parental line was used, one recognized as having a high level of SI, there were many differences in the F<sub>1</sub> purity from year to year in the seed production fields. Cruciferous plants have entomophilous flowers that can be pollinated by insects in the F<sub>1</sub> seed production fields. Taking all this into consideration, and using radish as experimental material, in the study presented here we have attempted an insect-pollination SI test to strictly evaluate the level of SI as a model for actual F<sub>1</sub> seed production field. In addition, we have tried

Communicated by H.F. Linskens

A. Horisaki · N. Tanaka · S. Niikura (✉)  
Tohoku Seed Co., Himuro, Utsunomiya, Tochigi, 321-3232, Japan  
e-mail: breeding@tohokuseed.co.jp  
Fax: +81-28-6676802

to clarify the relationship between artificial self-pollination and insect-pollination SI tests and that between the *S*-gene and the level of SI evaluated in both methods. We then performed a genetic analysis using the F<sub>2</sub> and F<sub>3</sub> population from which the level of SI would be expected to segregate. The effectiveness of the insect-pollination SI test over the artificial self-pollination test is discussed.

## Materials and methods

Twelve inbred lines of *Raphanus Sativus* L. homozygous for the *S*-alleles were used. In the artificial self-pollination test, the rate of self-seed setting was calculated from the number of pods bearing seed or seeds/the number of pollinated flowers. For the insect-pollination SI test, each line was sown, nursed and planted in separate isolated net tents. After their bolting and flowering, their self and/or sib pollens were gathered randomly by honey bees from hives set in each tent. When the flowering ended, each yield was examined on a volume basis.

In the genetic analyses, an F<sub>2</sub> population (97–520F<sub>2</sub>) and F<sub>3</sub> lines (97–520F<sub>3</sub>) derived from a cross (97–520F<sub>1</sub>) between R00-4 (high level of SI and homozygous for *S*<sup>208</sup>) and LV339 (low level of SI and homozygous for *S*<sup>210</sup>) were used. The F<sub>3</sub> seeds were obtained by selfing *S*-heterozygous (*S*<sup>208</sup>*S*<sup>210</sup>) plants randomly picked from 97–520F<sub>2</sub> populations by artificial bud pollination on main-stem flowers covered with paper bags before the bee hives were established. After the self-pollinations, these flowers were re-covered with the bags to prevent contamination by other pollen. The *S*-genotypes of all these plants were identified by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (Niikura and Matsuura 1998, 2001) at their nursing stage. The plants of each genotype (*S*<sup>208</sup>*S*<sup>208</sup>, *S*<sup>208</sup>*S*<sup>210</sup>, *S*<sup>210</sup>*S*<sup>210</sup>) were planted in separate, isolated net tents.

In a 1999 experiment, three R00-4, two LV339, three F<sub>1</sub> plants and 41 F<sub>2</sub> plants were sown on January 20, then planted out on February 23. The flowering period was between April 15 and early June. In a 2000 experiment, four R00-4, four LV339, five F<sub>1</sub> plants, 54 F<sub>2</sub> plant and five F<sub>3</sub> lines (each line consisting of 9–19 plants) were sown on November 26, 1999 and planted out on February 9, 2000. The flowering period was between March 30 and late May. The flowering period of the parental lines was almost the same. After the flowering ended, the yield and rate of self-seed setting were examined. The rate of self-seed setting was identified as the average calculated from the number of pods bearing seeds/total flowers on ten branches picked from the first to fourth branches. The recombination value was calculated with the maximum likelihood method (Allard 1956).

In an F<sub>1</sub> seed production test using R00-4 (high level of SI) as its parental line and insect pollination, the F<sub>1</sub> purity and yield of the harvested seeds from R00-4 was 100% (120 plants in sampling tests) and 65.5 ml per plant. The other parental line's yield was 56.0 ml per plant. These indicated that R00-4 was good enough for both male and female fertilization.

## Results

The relationship among *S*-genes, the rate of self-seed setting by artificial self-pollination and the yield obtained with the insect-pollination SI test.

Table 1 shows the *S*-allele, the rate of self-seed setting by artificial self-pollination and yields using the insect-pollination SI test. The rate of self-seed setting by artificial self-pollination in the inbred lines ranged from 0.00 to 0.60. The yield obtained by the insect-pollination

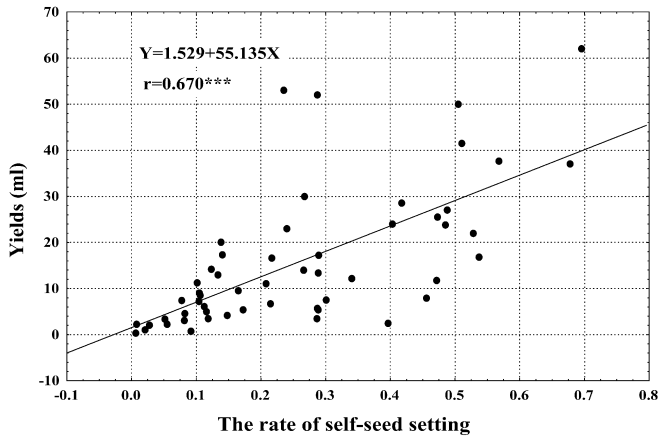
**Table 1** Comparison among the *S*-gene, the rate of self-seed setting (RSS) by the artificial self-pollinations and seed yield obtained by the insect-pollination SI test (*n.d.* not determined)

Accession no.	<i>S</i> -allele	RSS	Yield (ml)
R98-hL84	201	n.d.	5.2
R00-h301	201	n.d.	46.7
R00-23	201/213	n.d.	2.0
R00-133	202	0.00	38.0
R00-h177	203	0.33	48.0
R00-hL64	203	0.25	60.0
R00-h511	204	n.d.	21.6
R00-hL96	205	n.d.	1.3
R00-h30	205	n.d.	23.5
R00-h55	205	n.d.	50.0
R00-1	206	0.04	23.0
R00-h158	206	0.20	26.0
R00-h326	206	0.20	8.0
R00-h66	207	0.20	32.0
R00-h59	207	n.d.	32.0
R00-hV2B	207	n.d.	0.9
R00-4	208	0.00	2.0
R00-h83	208	n.d.	1.6
R00-h115	208	0.20	66.0
R00-hL14	208	n.d.	2.9
R00-hL120	208	0.00	2.5
R00-h350	210	n.d.	40.0
LV339	210	0.45	17.5
R00-hL158	212	n.d.	20.0
A88	213	0.40	28.0
R00-3	213	0.60	2.0
R00-hL46	225	n.d.	27.0
R00-hL59	226	0.20	76.0

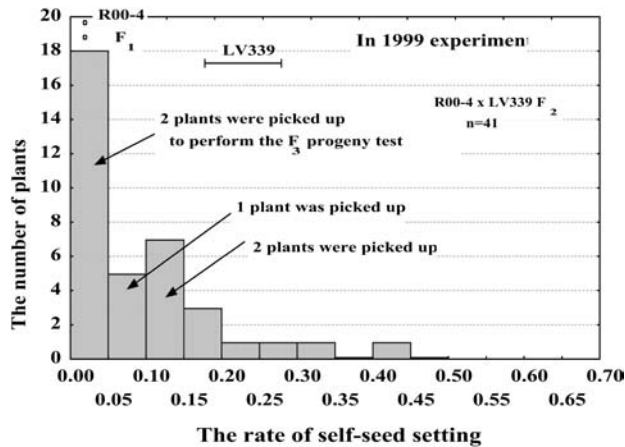
SI test ranged from 0.9 ml to 76.0 ml. Notice that lines showing low yields are recognized as high-level SI lines, while lines showing high yields are recognized as low-level SI lines. The yields of R00-h177 and R00-hL64 (*S*<sup>203</sup>-homozygotes) were very high. However, there was a wide range in yields among lines homozygous for *S*<sup>206</sup> or *S*<sup>208</sup>. There was a positive correlation ( $r = 0.606$ ) between the rate of self-seed setting by the artificial self-pollination and the yield obtained by the insect-pollination SI test. R00-1 and R00-133 showed high yields in the insect-pollination SI test despite showing a low rate of self-seed setting in the artificial self-pollination. On the other hand, no lines showing a high rate of self-seed setting in the artificial self-pollination had low yields in the insect-pollination SI test.

## Genetic analysis of the level of SI evaluated by the insect-pollination

It is possible that yield is influenced by plant or seed size. Thus, to select a more exact evaluation of level of SI characteristics in the insect-pollination SI test, we examined the relationship between yield and the rate of self-seed setting in the F<sub>2</sub> population (Fig. 1). There was a high positive correlation ( $r = 0.670$ ) between these characteristics. We adopted here this rate of self-seed setting as a criterion of level of SI in the insect-pollination SI test.

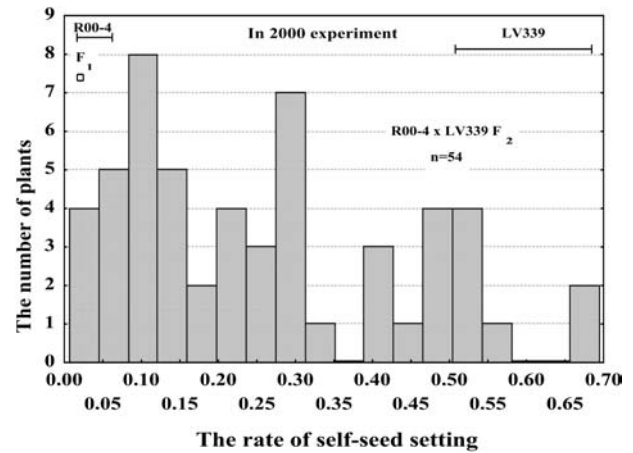


**Fig. 1** Relationship between the yields and the rate of self-seed setting by insect pollination in the  $F_2$  population derived from the cross R00-4, high-level SI line, and LV339, low-level SI line

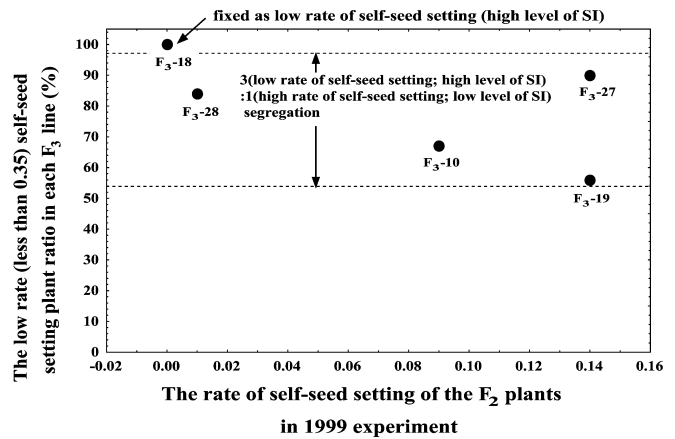


**Fig. 2** Frequency distribution of the rate of self-seed setting by insect pollination in the  $F_2$  population derived from the cross R00-4, high-level SI line, and LV339, low-level SI line, in 1999 experiment

We studied the mode of inheritance of the rate of self-seed setting in the  $F_2$  population. In a 1999 experiment (Fig. 2), the average rate of self-seed setting of R00-4, showing high-level SI, was  $0.00 \pm 0.00$  (S.D.); the low-level SI line, LV339, showed  $0.23 \pm 0.06$ . The average rate of self-seed setting of the  $F_1$  plants crossed with these parental lines was  $0.03 \pm 0.01$ . The rate of self-seed settings of the  $F_2$  population ranged variously from 0.00 to 0.45. In a 2000 experiment (Fig. 3), the average rate of self-seed setting of R00-4 was  $0.04 \pm 0.02$ ; LV339 showed  $0.60 \pm 0.07$ . The average rate of self-seed setting of the  $F_1$  plants crossed with these parental lines was  $0.01 \pm 0.00$ . The rate of self-seed settings of the  $F_2$  population showed an extensive segregation from 0.00 to 0.70 as well as a binomial or trinomial distribution. Our criterion for separating the population showing a low rate of self-seed setting (high level of SI) and one showing a high rate of self-seed setting population (low level of SI) was the



**Fig. 3** Frequency distribution of the rate of self-seed setting by insect pollination in the  $F_2$  population derived from the cross R00-4, high-level SI line, and LV339, low-level SI line, in 2000 experiment



**Fig. 4** Relationship between the rate of self-seed setting by insect pollination of randomly selected  $F_2$  plants from the cross R00-4, high-level SI line, and LV339, low-level SI line, in 1999 experiment, and the low rate (less than 0.35) of self-seed setting plant ratio of the  $F_3$  lines derived from these  $F_2$  plants. The rate of self-seed setting segregation of the  $F_3$  lines plotted between two dashed lines fit the 3 (low rate, high level of SI):1 (high rate, low level of SI) ratio at the 5% level of probability

distribution gap at 0.35. There were 39 high-level SI plants and 15 low-level SI plants, which fit a segregation ratio for the level of SI of 3 (high level of SI):1 (low level of SI) well. This result suggested that the high level of SI which R00-4 showed is controlled by a dominant gene, tentatively designated *HLSI-1* (high level of SI). We performed recombination analysis between *HLSI-1* and the *S*-gene as a data set of 9:3:3:1 because *S*-gene segregation varied from an expected ratio (1:2:1) and thus was thought to be 3 ( $S^{208}$  homozygotes and  $S^{210}S^{208}$  heterozygotes):1 ( $S^{210}$  homozygotes). The calculated recombination value was  $27.2 \pm 7.3$  (Table 2).

A progeny test was performed using five  $F_3$  lines that originated from the selfing of plants randomly selected

**Table 2** Combined segregation between the *S*-gene and *HLSI-1* (high level of *SI-1*) in an F<sub>2</sub> population derived from the cross between R00-04 and LV339

Linkage phase	Calculated recombination value (%)		F <sub>2</sub> population				Total	Goodness of fit		
			<i>S</i> <sup>208</sup> <i>S</i> <sup>208</sup> <i>S</i> <sup>208</sup> <i>S</i> <sup>210</sup> <i>HLSI-1</i>	<i>S</i> <sup>210</sup> <i>S</i> <sup>210</sup> <i>HLSI-1</i>	<i>S</i> <sup>208</sup> <i>S</i> <sup>208</sup> <i>S</i> <sup>208</sup> <i>S</i> <sup>210</sup> +	<i>S</i> <sup>210</sup> <i>S</i> <sup>210</sup> +		Ratio	χ <sup>2</sup>	<i>P</i>
Coupled	27.2 ± 7.3	Observed	33	6	7	8	54	9:3:3:1	9.211	<0.05
		Calculated	34.16	6.35	6.35	7.16	54.00		0.226	0.90–0.99

**Table 3** The number of plants examined, the rate of self-seed setting (RSS) at their F<sub>2</sub> generation, the average RSS, their standard deviation (S.D.) and assumed *HLSI-1* genotype in each F<sub>3</sub> line

	Number	Number of plants	RSS at F <sub>2</sub> generation	RSS at F <sub>3</sub> generation		<i>HLSI-1</i> genotype
				Average	S.D.	
F <sub>3</sub> -18	9	9	0.00	0.09	0.09	Homozygous
F <sub>3</sub> -28	19	19	0.01	0.17	0.15	Heterozygous
F <sub>3</sub> -10	18	18	0.09	0.30	0.18	Heterozygous
F <sub>3</sub> -19	9	9	0.14	0.44	0.25	Heterozygous
F <sub>3</sub> -27	10	10	0.14	0.16	0.12	Heterozygous

from the F<sub>2</sub> population (Fig. 4, Table 3). The relationship between the rate of self-seed setting of the F<sub>2</sub> plants and the low rate of self-seed setting plant ratio in each F<sub>3</sub> line showed the following two patterns: one F<sub>3</sub> line determined to be a low rate of self-seed setting; four F<sub>3</sub> lines varied from a low to a high rate of self-seed setting and fit the 3 (low rate of self-seed setting, that is high level of *SI*):1 (high rate of self-seed setting; that is low level of *SI*). No F<sub>3</sub> lines were observed to fix as a high rate of self-seed setting because no plants showing the high rate of self-seed setting were isolated from the F<sub>2</sub> population.

## Discussion

We had already reported that there is no relationship between the *S*-gene and the rate of self-seed setting as determined by the artificial pollination tests (Niikura and Matsuura 1999). In this insect-pollination *SI* test, we showed that the wide variation of yields within the lines having the same *S*-allele suggests the same as the aforementioned. *HLSI-1* was also found to be loosely linked to the *S*-gene (calculated recombination value: 27.2 ± 7.3%). The results of this study confirmed that the genes related to the level of *SI* are not the *S*-gene. We had already isolated a PCR product common to low-level *SI* lines by a simple differential display method (Yoshida et al. 1994). The PCR product sequence had a high homology with *S*-adenosyl methionine synthase (Niikura 2002). Furthermore, Northern blot analysis using this PCR product as a probe showed that the faint transcript was also detected in the high-level of *SI* lines. This result indicated that a regulatory gene of transcript originated from this PCR product is a trigger. It is necessary to isolate this type of gene for further study.

The insect-pollination *SI* test is thought to be a more reliable and stricter method than the artificial self-pollination test with respect to an evaluation of *SI* level.

This is because there was a positive correlation between these two test results and because R00-1 and R00-133 showed a high yield in the insect-pollination *SI* test despite showing low rate of self-seed setting in artificial self-pollination. On the other hand, no lines showing a high rate of self-seed setting showed a low yield as determined by the insect-pollination *SI* test. Why did R00-1, R00-133 show a high yield in the insect-pollination *SI* test despite showing a low rate of self-seed setting in the artificial self-pollination? The situation of the insect-pollination *SI* test, compared with that of artificial self-pollination test, is as follows: a flower (stigma) can be pollinated, its self-pollen mixed with sib-pollen by the insects honey bees in this study and this can occur repeatedly from the opening of the flower to falling. An entire plant will also be pollinated from the beginning to the end of the flowering period. The level of *SI* tends to decline as the plant ages (Stout 1920). The pollinating stimulation by the insects will be added to the situation. The additive effect and/or interaction among these or other unknown factors result in the strictness of the insect-pollination *SI* test. The average rate of self-seed setting of LV339 (low-level *SI*), F<sub>1</sub> plants and F<sub>2</sub> population in 1999 was lower than that in the 2000 experiment. This was probably because later flowering and a smaller net tent in 1999, which led to a decline in fertilization ability and honey bees' activity by being exposed to higher temperatures – especially in the latter one-third of the flowering period, the average high temperature was 26.3 °C in 1999 and 23.3 °C in 2000. If the expressivity of *SI* level in the 2000 experiment had been stricter and more accurate, the high level of *SI* of F<sub>1</sub> plants, and the segregation ratio – 3(high-level *SI*):1(low-level *SI*) in the F<sub>2</sub> population – might have suggested that a gene governing the high-level *SI* of R00-4 was a dominant gene, *HLSI-1*. There was a trend that the lower the rate of self-seed setting in a plant in the F<sub>2</sub> population, the lower the rate of self-seed setting in their F<sub>3</sub> progenies. This result also confirms that *HLSI-*

*I* would act as a dominant gene and suggests that the high level of SI can be fixed by early generation selection. We did not examine the reproductive success rate of ovules in pollinated flowers (Namai and Ohsawa 1986) in this experiment. There was another distribution peak in the high level of SI plants in the F<sub>2</sub> population. This peak might show the segregation of variation in the reproductive success rate of ovules in pollinated flowers.

The products from both stigma and pollen from the *S*-gene have been identified owing to the advances of molecular analysis (Watanabe et al. 2001). It is not too much to say that a part of study for the self-incompatibility ended because so-called self-incompatibility includes the *S*-gene governing the sporophytic SI aforementioned, modifier gene (Ikeda et al. 1997), gametophytic SI (Zuberi and Lewis 1988), seed abortion and sexual selection (Marshall and Ellstrand 1986, 1988), heteromorphic incompatibility, and so on. In addition to this, for F<sub>1</sub> seed production, it is also important to understand the control of flowering because it is necessary to synchronize the flowering time of its parental lines. Thus, we must pay attention to these phenomena to make the whole of so-called SI clear in the future. This will lead to the stable supply of seeds with high F<sub>1</sub> purity to farmers throughout the world.

**Acknowledgements** We thank Dr. Y. Fujita, an executive director of Tohoku Seed Company, for his support and Dr. S. Matsuura of Tohoku Seed Company for his generous advise during this study.

## References

- Allard RW (1956) Formulas and tables to facilitate the calculation of recombination value in heredity. *Hilgardia* 24:235–279
- Bateman AJ (1952) Self-incompatibility systems in angiosperms. (I.) Theory. *Heredity* 6:285–310
- Bateman AJ (1955) Self-incompatibility systems in angiosperms. (III). Cruciferae. *Heredity* 9:52–68
- Göring DR, Glavin TL, Schäfer U, Rothstein SJ (1993) An *S* receptor kinase gene in self-compatible *Brassica napus* has a 1-bp deletion. *Plant Cell* 5:531–539
- Ikeda S, Nasrallah JB, Preiss RDS, Nasrallah ME (1997) An aquaporin-like gene required for the *Brassica* self-incompatibility response. *Science* 276:1564–1566
- Good SV, Stephenson AG (2002) The inheritance of modifiers conferring self-fertility in the partially self-incompatible perennial, *Campanula rapunculoides* L. (CAMPANULACEAE). *Evolution* 56(2):263–272
- Marshall DL, Ellstrand NC (1986) Sexual selection in *Raphanus sativus*: experimental data on nonrandom fertilization, maternal choice, and consequences of multiple paternity. *The American Naturalist* 127:446–461
- Marshall DL, Ellstrand NC (1988) Effective mate choice in wild radish: evidence for selective seed abortion and its mechanism. *The American Naturalist* 127:446–461
- Namai H, Ohsawa R (1986) Variation of reproductive success rates of ovule and pollen deposited upon stigmas according to the different number of pollen on a stigma in angiosperm. In: Mulcahy DL, Mulcahy GB, Ottaviano E (eds) *Biotechnology and Ecology of Pollen*, Springer-Verlag, New York, pp 63–68
- Niikura S, Matsuura S (1997) Genomic sequence and expression of *S*-locus gene in radish (*Raphanus sativus* L.). *Sex Plant Reprod* 10:250–252
- Niikura S, Matsuura S (1998) Identification of self-incompatibility alleles (*S*) by PCR-RFLP in radish (*Raphanus sativus* L.). *Euphytica* 102:379–384
- Niikura S, Matsuura S (1999) Genetic variation of the *S*-alleles and level of self-incompatibility in the Japanese cultivated radish (*Raphanus sativus* L.) (in Japanese). *Breed Res* 1:211–220
- Niikura S, Matsuura S (2000) Genetic analysis of the reaction level of self-incompatibility to a 4% CO<sub>2</sub> gas treatment in the radish (*Raphanus sativus* L.). *Theor Appl Genet* 101:1189–1193
- Niikura S, Matsuura S (2001) Genetic variation of the self-incompatibility alleles (*S*-alleles) in the cultivated radish (*Raphanus sativus* L.) by the PCR-RFLP method. *Acta Hort* 546:359–365
- Niikura S (2002) Practical studies of the self-incompatibility on vegetable seed breeding and production in the cruciferous vegetables. *Proceedings of International Workshop Seed & Seedling Science & Technology*, Taiwan. Food and Fertilizer Technology Center for the Asian and Pacific Region
- Ruffio-Chable V, Herve Y, Dumas C, Gaude T (1997) Distribution of *S*-haplotypes and its relationship with self-incompatibility in *Brassica oleracea*. Part 1. In inbred lines of cauliflower (*B. oleracea* var 'botrytis'). *Theor Appl Genet* 94:338–346
- Stout AB (1920) Further experimental studies on self incompatibility in hermaphroditic plants. *J Genet* 9:85–129
- Tantikanjana T, Nasrallah ME, Stein JC, Chen CH, Nasrallah JB (1993) An alternative transcript of the *S* locus glycoprotein gene in a class II pollen-recessive self-incompatibility haplotype of *Brassica oleracea* encodes a membrane-anchored protein. *Plant Cell* 5:657–666
- Watanabe M, Hatakeyama K, Takada Y, Hinata K (2001) Molecular aspects of self-incompatibility in *Brassica* species. *Plant Cell Physiol* 42:560–565
- Yoshida K, Naito T, Takeda G (1994) cDNA cloning of regeneration-specific genes in rice by differential screening of randomly amplified cDNAs using RAPD primers. *Plant Cell Physiol* 35:1003–1009
- Zuberi MI, Lewis D (1988) Gametophytic-sporophytic incompatibility in the Cruciferae-*Brassica campestris*. *Heredity* 61:367–377