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S. Niikura · S. Matsuura

Genetic analysis of the reaction level of self-incompatibility to a 4% CO_2 gas treatment in the radish (*Raphanus sativus* L.).

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Abstract In radishes, self-incompatibility (SI) is governed by the *S*-locus, which consists of a series of multiple alleles. This SI can be overcome by CO_2 gas treatment, a characteristic that is very useful in obtaining large amounts of parental seeds for F_1 commercial seeds. We know from experience that there are genetic variations in the reaction level of self-incompatibility (RLSI) to a 4% CO_2 gas treatment (hereafter described as RLSICO_2) in the radish. We have raised and analyzed an F_2 population derived from an F_1 cross between No. 9324 (S^{206}-homozygote, low RLSICO_2) and LV364 (S^{209}-homozygote, high RLSICO_2). The RLSICO_2 among three *S*-genotypes (S^{206}-homozygotes, $S^{206}S^{209}$-heterozygotes, S^{209}-homozygotes), which fit the theoretical ratio of one gene segregation in the F_2 population, did not show any significant statistical differences. Hence, we concluded that the RLSICO_2 was controlled by a gene other than the *S*-gene. In this F_2 population the segregation of the RLSICO_2 fit the 3(low RLSICO_2):1(high RLSICO_2) ratio well. This result and F_3 progeny tests suggest that high RLSICO_2 is controlled by a recessive gene. Reciprocal crosses among S^{209}-homozygotes with different RLSICO_2 have shown that this gene would act in the stigma.

Keywords CO_2 gas treatment · Parental seeds · *Raphanus sativus* · *S*-gene · The reaction level of self-incompatibility

Introduction

In cruciferous plants, self-incompatibility (SI) is governed by the *S*-locus, which consists of a series of multiple alleles. The family of radishes belongs to the family *Cruciferae* and also exhibits sporophytic SI with multiple *S*-alleles located at a single locus (Bateman 1955). Some dozens of *S*-alleles have been identified to date (Okazaki and Hinata 1984; Lewis et al. 1988; Karron et al. 1990). Genetic variations in the levels of SI (LSI) in the Brassicas have been reported (Haruta 1962; Ruffio et al. 1997) including *Raphanus*. In addition, the SI can be overcome easily by several external factors, such as organic solvents (Tatebe 1968), NaCl (Tao and Yang 1986; Monterio and Gabelman 1988) and high temperature (Matsubara 1980; Okazaki and Hinata 1987).

As well as the above treatments SI can also be overcome by a CO_2 gas treatment, as in *Brassica campestris* (Nakanishi et al. 1969). The effective density, timing and humidity for this CO_2 gas treatment have been clarified in *B. oleracea* and *B. campestris* (Nakanishi and Hinata 1973; Nakanishi and Hinata 1975; Dhaliwal et al. 1981; Palloix et al. 1985). Nakanishi and Hinata (1973) also showed the existence of a genetic variation in the reaction level of SI (RLSI) to a 4% CO_2 gas treatment (hereafter described as RLSICO_2) in *Brassica*. However, there have been no studies to date either on the elucidation of the genetic relationship between the *S*-gene and RLSICO_2 or as to the mode of inheritance of the RLSICO_2, both of which are very important to the breeding of these cruciferous crops.

In radish, we also know from our experience using a 4% CO_2 gas treatment that there are genetic variations in the RLSICO_2. Here we will describe the relationship between the *S*-alleles and RLSICO_2 as well as a subsequent genetic analysis of the RLSICO_2 using these radishes.

Materials and methods

Plant materials

We have identified 37 *S*-alleles, tentatively designated S^{201} to S^{237} (Niikura and Matsuura 1999). Thirty-four local varieties homozygous for the *S*-locus (S^{202}, S^{203}, S^{209}, S^{210}, S^{216}, S^{218}, S^{219} and S^{230}) of the radishes, 128 F_2 plants of the cross No.9324 (S^{206}-homozy-

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S. Niikura (✉) · S. Matsuura
Tohoku Seed Co., Himuro, Utsunomiya, Tochigi, 321-3232, Japan
Fax: +81-28-667-6802
e-mail: tohokuis@mb.infoweb.ne.jp

gote, low RLSICO$_2$)×LV364 (S^{209}-homozygote, high RLSICO$_2$) and 12 F_3 lines consisting of 16 plants randomly selected from the F_2 population were used for this study. The above materials were vernalized at 5°C for 2 weeks in a refrigerator and then grown in a greenhouse. The experiments using local varieties and the F_2 population were performed in 1997. The F_3 progeny tests were carried out in 1999.

Determination of *S*-genotypes

We had previously cloned the *S*-locus gene in the radishes (Niikura and Matsuura 1997). We subsequently succeeded in applying the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to them (Niikura and Matsuura 1998). In the experiments to determine the *S*-genotypes, this method was performed on the test crosses of each plant of the F_2 population. In the test crosses, the pollinated flowers were dissected out and placed on an agar medium. After 1 day at room temperature, the other floral organs were removed, leaving the pistils which were then softened with a NaOH solution, stained with aniline blue according to the Kho and Baer method (1968) and observed under a fluorescent microscope. Two flowers were used in each cross combination. Pollen-tube behavior was observed under a fluorescent microscope. Compatibility was based on the growth of pollen tubes into the stigmas; i.e. cross combinations showing 4 or more penetrating pollen tubes were judged as 'compatible(+)' and those showing fewer than 4 as 'incompatible(–)'.

Evaluations of RLSICO$_2$

Two self-pollinated flowers in anthesis from each plant were dissected out and placed on an agar medium. Following this, these flowers were subjected to a 4% CO_2 gas treatment for 4 h at 25°C in a CO_2 incubator at high relative humidity. After 1 day, their pistils were softened, stained and observed as described above. The RLSICO$_2$ was classified into 5 categories, based on the number of pollen tubes penetrating into the stigmas: (1) 0–4; (2) 5–7; (3) 8–10; (4) 11–20; (5) more than 20. Three to five replicates were performed on each plant on other days with differing weather conditions. In control experiments performed as above but without CO_2 gas treatment, we always confirmed that no pollen tubes penetrated into the stigmas.

Table 1 Relationship between the *S*-alleles and reaction level of self-incompatibility to CO_2 (RLSICO$_2$) using local varieties of radishes

Accession no.	*S*-allele	RLSICO$_2$
LV 367aa	S^{202}	1.00
LV 302	S^{202}	1.25
LV 346	S^{202}	5.00
LV 387	S^{202}	5.00
LV 375	S^{203}	1.00
LV 331	S^{203}	1.75
LV 261	S^{209}	1.00
LV 364	S^{209}	4.25
LV 396	S^{210}	1.00
LV 329	S^{210}	2.50
LV 377	S^{210}	3.00
LV 339	S^{210}	4.00
LV 282bb	S^{216}	1.00
LV 17	S^{216}	1.50
LV 274	S^{216}	3.00
LV 372	S^{216}	3.00
LV 348	S^{216}	4.50
LV 31aa	S^{218}	1.00
LV 300	S^{218}	1.00
LV 275aa	S^{218}	4.00
LV 365	S^{218}	5.00
LV 164bb	S^{219}	1.00
LV 362	S^{219}	1.00
LV 315	S^{219}	1.75
LV 26aa	S^{219}	2.00
LV 363aa	S^{219}	2.50
LV 282aa	S^{219}	3.00
LV 379aa	S^{219}	3.50
LV 336	S^{230}	1.00
LV 347	S^{230}	1.00
LV 295	S^{230}	2.50
LV 380	S^{230}	4.50
LV 275bb	S^{230}	3.50
LV 279aa	S^{230}	5.00

Results

Relationship between the *S*-alleles and RLSICO$_2$

We first examined the RLSICO$_2$ of the local varieties whose *S*-alleles had been identified (Table 1). The 2 lines belonging the S^{203} group showed a low RLSICO$_2$, while in all the other *S*-allele groups, the lines showed a low to high RLSICO$_2$. Moreover, the average RLSICO$_2$ among the *S*-alleles did not show any significant statistical differences. We then raised and analyzed an F_2 population derived from an F_1 cross between No.9324 (S^{206}-homozygote, low RLSICO$_2$) and LV364 (S^{209}-homozygote, high RLSICO$_2$). Three *S*-genotypes (S^{206}-homozygotes, S^{206} S^{209}-heterozygotes, S^{209}-homozygotes) fit the theoretical ratio of a one-gene segregation in the F_2 population (Table 2). However, the average RLSICO$_2$ among these *S*-genotypes also did not show any significant statistical differences (Table 3). These results indicated that the RLSICO$_2$ was not controlled by the *S*-gene but by some other gene.

Genetic analysis of the RLSICO$_2$

We studied the mode of inheritance of the RLSICO$_2$ in the F_2 population. The average RLSICO$_2$ of No. 9324

Table 2 F_2 segregation of *S*-genotypes and average of the reaction level of self-incompatibility to CO_2 (RLSICO$_2$) in each *S*-genotype of the cross No. 9324×LV364

S-genotype	$S^{206}S^{206}$	$S^{206}S^{209}$	$S^{209}S^{209}$	Total	Goodness of fit		
					Ratio	χ^2	*P*
Observed	29	72	27	128	1:2:1	2.06	0.30–0.50
Calculated	32.00	64.00	32.00	128.00			
Average of RLSICO$_2$	1.96	1.92	1.96				

Table 3 Analysis of variance of the reaction level of self-incompatibility to CO_2 ($RLSICO_2$) among three *S*-genotypes in the F_2 population of the cross No. 9324×LV364 (*ns* non-significant)

Source	*df*	SS[a]	MS[b]	*F*
Total	127	136.50		
Genotype	2	0.06	0.03	0.03 ns
Error	125	136.44	1.09	

[a] Sum of square, [b] Mean square

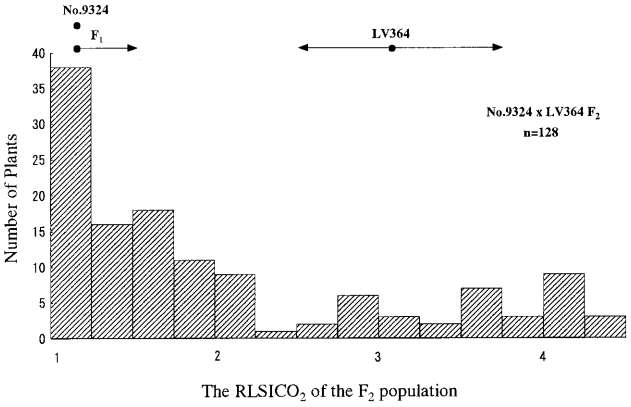

Fig. 1 Frequency distribution of the reaction level of self-incompatibility by CO_2 ($RLSICO_2$) in the F_2 population of the cross. No. 9324×LV364

showing low $RLSICO_2$ was 1.21±0.21 (±SD); the high $RLSICO_2$ line, LV364, showed 3.13±0.60. The average $RLSICO_2$ of the F_1 plants crossed between these parental lines was 1.07±0.15, which was almost the same as that of the low $RLSICO_2$ line, No. 9324. The $RLSICO_2$ of the F_2 population showed an extensive segregation from 1.00 to 4.25, as well as a binomial distribution (Fig. 1). Our criteria for separating the low $RLSICO_2$ population and the high $RLSICO_2$ population were the distribution gap at 2.25–2.49 and the Standard deviation range of LV364. There were 92 low $RLSICO_2$ and 36 high $RLSICO_2$ plants, which fit a segregation ratio for the $RLSICO_2$ of 3 (low $RLSICO_2$):1 (high $RLSICO_2$) well

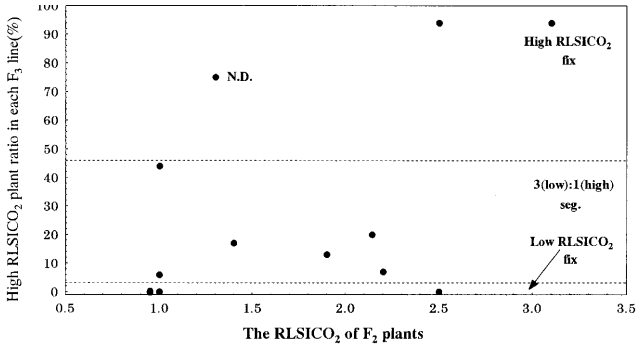

Fig. 2 Relationship between the reaction level of self-incompatibility by CO_2 ($RLSICO_2$) of randomly selected F_2 plants from the cross No. 9324×LV364, and the high $RLSICO_2$ plant ratio of the F_3 lines derived from these F_2 plants. The $RLSICO_2$ segregation of the F_3 lines plotted between two *dashed lines* fit the 3 (low $RLSICO_2$):1 (high $RLSICO_2$) ratio at the 5% level statistically. *N.D.* Not determined

(Table 4). This result suggested that high $RLSICO_2$ is controlled by a recessive gene. We performed a progeny test using 12 F_3 lines randomly selected from the F_2 population. The relationship between the $RLSICO_2$ of the F_2 plants and the high $RLSICO_2$ plant ratio in each F_3 line is shown in Fig. 2. This figure shows the following three patterns: 3 F_3 lines fixed as low $RLSICO_2$; 6 F_3 lines that varied from low to high $RLSICO_2$ and fit the 3 (low $RLSICO_2$):1 (high $RLSICO_2$) ratio; 2 F_3 lines fixed as high $RLSICO_2$. One F_3 line, however, could not be classified into any group. The segregation of the other F_3 lines fit the theoretical ratio of F_3 line segregation for one gene (Table 5) and also confirmed that this gene would act as a recessive gene.

Expressed organ of the gene which governs high $RLSICO_2$

To examine where the gene governing high $RLSICO_2$ acts, we used 3 S^{209}-homozygotes with different $RLSICO_2$ from the F_2 population: F_2–1, low $RLSICO_2$;

Table 4 F_2 segregation of the reaction level of self-incompatibility to CO_2 ($RLSICO_2$) in the cross No. 9324×LV364

	$RLSICO_2$		Total	Goodness of fit		
	Low	High		Ratio	χ^2	*P*
Observed	92	36	128	3:1	0.67	0.30–0.50
Calculated	96.00	32.00	128.00			

Table 5 F_3-line segregations of the reaction level of self-incompatibility to CO_2 ($RLSICO_2$) randomly selected F_2 plants of the cross No. 9324×LV364

$RLSICO_2$	Low fixation	3 (low):1 (high) seg.	High fixation	Not determined	Total	Goodness of fit		
						Ratio	χ^2	*P*
Observed	3	6	2	1	12	1:2:1	0.27	0.70–0.90
Calculated	2.75	5.50	2.75		11.00			

Table 6 Reciprocal crosses among three *S*^{209}-homozygotes with different reaction levels of self-incompatibility to CO_2 ($RLSCO_2$) selected from the F_2 population of the cross No. 9324×LV364. Control group is without CO_2 gas treatment; CO_2 group (CO_2) is with a 4% CO_2 gas treatment

♀/♂	F_2-1	F_3-31	F_2-55
Control			
F_2-1 (Low)[a]	–[b]	–	–
F_2-31 (High)	–	–	–
F_2-55 (High)	–	–	–
CO_2			
F_2-1 (Low)	–	–	–
F_2-31 (High)	+[b]	+	+
F_2-55 (High)	+	+	+

[a] Low, low $RLSCO_2$; High, high $RLSCO_2$
[b] +, Many pollen tubes penetrating; –, no pollen tubes penetrating

F_2–31, high $RLSICO_2$; F_2–55, high $RLSICO_2$. Reciprocal crosses were performed among these plants, but only when F_2–31 and F_2–55 were used as the female did the cross combinations show high $RLSICO_2$ (Table 6). This means that this gene would act in the stigma.

Discussion

Cultivated radishes are the most common vegetables in the world, especially in eastern Asia, with one of the largest areas under cultivation and a large number of local varieties. Recently, most of the radish cultivars have been F_1 hybrids and the hybrid seeds are produced using an SI system. Thus, in the case of large-scale commercial F_1 hybrid seed production, vast amounts of parental seeds are needed to be commensurate with F_1 seed quantity. The methods for parental seed production are bud pollination, the double crossing method (Haruta 1962), using a pair of isogenic lines with respect to *S*-alleles and overcoming SI by a NaCl solution (Tao and Yang 1986; Monterio and Gabelman 1988) or a CO_2 gas treatment. Bud pollination is laborious and involves high costs. Radish breeders have usually adopted the double crossing method when they need larger amounts of parental seeds to reduce their costs. The NaCl treatment is simple and adequate for small-scale seed production, but it is not used frequently because it is detrimental to pollinating insects. The CO_2 gas treatment can be used uniformly on a large scale. Hence, in the case of using parental lines showing high $RLSICO_2$ and of having to produce F_1 seed in a short time for some reason, many seed companies around the world adopt this method in treating the radish F_1 hybrids. These parental seed production methods are all requisite for radish breeding. We have further identified that the greater part of the LSI is also governed by genes other than the *S*-gene in the radish, as well as *B. oleracea* (Ruffio et al. 1997). We recognize that any line showing weak SI is apt to show high $RLSICO_2$. If the parental seeds of a line are produced repeatedly by CO_2 treatments, the line will tend to show low LSI. The use of a parental line showing low LSI in F_1 seed production results in a decrease in the purity of the hybrid seed lots. The key point in radish breeding is whether to separate these two characteristics genetically. An ideal parental line shows high $RLSICO_2$ in parental seed production but also shows strong SI in F_1 seed production. From this point of view, it is very important to clarify the genetic relationships among the *S*-gene, LSI and $RLSICO_2$.

We evaluated $RLSICO_2$ based on the degree of pollen-tube penetration into the stigma and were able to identify the genetic variability of $RLSICO_2$. These results suggest that there is no relationship between the $RLSICO_2$ and the *S*-gene and, from the F_2 and F_3 analyses, that high $RLSICO_2$ is governed by a recessive gene. These two findings are very significant for radish breeding. The breeder can, based on the former relationship, freely exchange one *S*-allele for another *S*-allele in the parental lines without limiting the sort of *S*-alleles existing in the breeding materials. This also means that they can diversify the variation of the cross combinations. Moreover, the latter result confirms that they can treat this characteristic as a major gene and fix high $RLSICO_2$ by the genetic selection in early generations.

A gene governing high $RLSICO_2$ was shown to be expressed in the stigma (Table 6). Thus, we tried to clone this gene by the differencial display method using arbitrary primers (Yoshida et al. 1994). We used 282 primers but could not obtain any polymorphisms between the low $RLSICO_2$ plants and high $RLSICO_2$ plants. If the gene does not express itself in the stigma at the flowering stage, i.e. if the CO_2 gas treatment does not induce this gene expression, SI would not be overcome through gene expression. One of the hydration steps in the compatibility development is the transfer of water from the stigma to the grain. The availability of water to the grain is regulated by the stigmas (Elleman and Dickinson 1994). Kanno and Hinata (1969) observed a change in the papilla cells at the attachment point of pollen tubes on self-pollinated stigmas. Given that CO_2 gas treatment decreases the pH of the conditions inside the incubator or greenhouse, in the acid condition, the osmotic pressure of papilla cell walls or vacuoles may change and the water may be released from the stigmas. On the other hand, it has been proposed that the CO_2 gas may also modify stigma carbohydrate metabolism (Palloix et al. 1985). Thus, this gene probably governs the construction and/or metabolism of the stigma, reacting to the acid condition indirectly or to the CO_2 gas directly without any gene expression.

We think that SI is expressed through a genetic hierarchy consisting of the *S*-gene and the other genes governing LSI, $RLSICO_2$ and so forth. To understand the mechanism of SI totally for the breeding of the Brassicas, it is necessary at first to grasp phenotypic expression properly and to elucidate the genetic relationships among the genes governing the LSI and reacting to the external factors overcoming SI as mentioned above. Our objective now is to examine the relationship between the $RLSICO_2$ and the LSI.

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