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Development of Uniform F_1 Hybrid Varieties of Korean Radish Using Self-Incompatibility in Double-Crossing

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ABSTRACT

Radish (*Raphanus sativus* L.) is one of the major vegetables comprising 'Kimchi', which is the general term given to a group of fermented vegetables in Korea. The radish for 'Kimchi' is different from the European radish, which is used mainly in salads, and will be referred to as 'Korean radish' hereafter. Radish possesses self-incompatibility (SI) which prevents self-fertilization by recognizing self-pollen on the stigmatic surface. SI has been effectively used to produce uniform F_1 hybrid seeds; however, SI has caused problems in seed propagation of parental lines. Radish also bears a small number of seeds per pod compared to Chinese cabbage. To produce parental seeds effectively, breeders have made double-crossed (DC) varieties using similar inbreds, even though DC varieties show low uniformity. We have tried to increase seed production and uniformity by using cross-compatible near-isogenic lines (CCNILs). We have modified the single seed descent method by employing DNA markers on the *S*-locus. Hundreds of inbreds have been developed from two parental sets after one parental cross and five generations of inbreds. In each generation, PCR-based selection has been performed to select SI heterozygotes. We have tried to make inbreds possessing a different SI allele but with other genes fixed. Ways to use CCNILs in radish breeding will be discussed.

Keywords: breeding, double cross, hybrid variety, marker-assisted selection, near-isogenic lines, purity

INTRODUCTION

Self-incompatibility (SI) prevents self-fertilization and promotes out-crossing in hermaphroditic plants (Nettancourt 1997). In *Brassica* crops, SI is controlled by a single, highly polymorphic *S*-locus consisting of a series of multiple alleles (Bateman 1955; Watanabe *et al*. 2006). SI has been used to produce seeds of uniform F_1 hybrid varieties by preventing self-pollination within the two parental inbred lines (Sakamoto *et al*. 2000). However, SI causes problems in seed production of parental inbred lines and development of inbred lines by prohibiting self-pollination. Thus ways of escaping or temporal breakdown of SI have been developed.

A high concentration (approx. 5%) CO_2 treatment was developed for the temporal breakdown of the SI response (Lee 1979; Park and Jung 1983). Using Chinese cabbage and radish, a minimum of 3% CO_2 gas was supplied through pipelines at night after day-time bee (*Apis mellifera* L.) pollination. The CO_2 treatment required a greenhouse to prevent it from escaping, a CO_2 supply system composed of underground pipelines and a control box, bees as pollinators, and ventilation labour before insertion of the pollinating bees (Lee 1979). The CO_2 treatment made it possible to produce considerable amounts of parental seed to create the F_1 hybrid varieties. With the help of a high CO_2 concentration treatment, the single cross breeding method has been widely used to develop varieties in *Brassica* vegetables in Korea. However, labor and facilities for CO_2 treatment raised the seed production cost. Radish (*Raphanus sativus* L.) bears a small number of seed per pod compared to other *Brassica* crops (Niikura and Matsuura 1998) such as Chinese cabbage (*Brassica rapa* L.), cabbage (*Brassica oleracea* L.), and mustard (*Brassic juncea* L.), which also raises the price of hybrid radish seeds.

Commercial breeders tried to produce hybrid seeds more effectively through the introduction of the double-cross method (DC) in radish. DC was proposed in cabbage and marrow stem kale (*Brassica oleracea* var. *medullosa*) to produce parental seeds using SI (Odland and Knoll 1950; Thompson 1959). To produce DC varieties in cabbage, parental materials were selected on the basis of their yield, quality, and disease resistance. The seeds of the parental materials were produced using two sets of cross-compatible inbred lines. However, the uniformity of the DC varieties was low. These low uniformity DC varieties were not acceptable as commercial varieties (Thompson 1959).

In the late 1970s, a Korean breeder in a commercial seed company tried to develop SC varieties in radish using a high CO_2 concentration treatment. Even though he encountered difficulties in seed production, he could manage to produce considerable amount of parental seeds. Before that period, land race or DC varieties were sold in seed market. Even though the first SC variety of 'Taebaek' radish was very expensive compared to other varieties, it became very popular among Korean farmers. After that, many SC varieties were developed by many commercial seed companies (Cho 2006). These days, most varieties of radish and Chinese cabbage have been developed using single cross breeding.

Because of the high seed production efficiency, commercial breeders have been trying to use double cross breeding by raising the uniformity of DC varieties by using very similar inbreds as parental lines. However, the uniformity of DC variety is low compared to that of SC. Until the present day, only small numbers of DC varieties have been developed in cheap minor small and leafy radish.

The SI response of *Brassica* is controlled by a single multi-allelic locus, the *S* locus (Bateman 1955). As the *S*

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locus is a locus complex, the classical term of '*S*-allele' was changed to '*S*-haplotype' (Nasrallah and Nasrallah 1993). The *S*-locus spans ~80-100 kb and contains as many as 17 genes (Watanabe *et al*. 2000, 2006). Among them, three tightly linked highly polymorphic genes are required for the SI response; these are the *SLG*, *SRK*, and *SP11/SCR* genes. The *SLG* gene encodes a highly polymorphic secretory glycoprotein synthesized abundantly in the stigma (Nasrallah *et al*. 1987; Boyes and Nasrallah 1993). The *SRK* gene encodes a transmembrane protein, which consists of an intracellular serine-threonine protein kinase, a transmembrane domain, and an extracellular domain showing a highly similar sequence with the *SLG* gene (Stein *et al*. 1991). The *S*-haplotypes of radish were identified using PCR-RFLP with *SLG*- and *SRK*-specific primers (Lim *et al*. 2002). With the help of DNA markers, we tried to improve the uniformity of DC varieties using cross-compatible near-isogenic lines (CCNILs) in radish. Without the DNA markers, developing CCNILs is almost impossible because selecting SI heterozygotes through conventional methods requires a tremendous amount of time and labour.

Single seed descent (SSD) is a method that enables plant breeders to advance generations while maintaining genetic variability (Johnson and Bernard 1962). The aim of the SSD procedure is to make many homozygous lines in a short time at a low cost. In soybean, SSD was found to be more efficient than pedigree selection for yield improvement. Most advanced populations of soybean varieties are derived from elite × elite crosses that reduce segregation for many traits of economic importance (Boerma and Cooper 1975). In radish, SSD was proven to be efficient for developing big spring varieties. Two spring varieties were crossed and 300 progenies were put through SSD. Among 240 F_6 plants, 78.3% of the inbred lines showed high uniformity and 45% bolted similar or later than the late bolting parent. After combining ability and seed-bearing tests, a late bolting and high yielding spring radish cultivar was developed (Lee and Yoon 1987). Thus, we used SSD as a breeding method while adapting DNA markers to select for SI heterozygotes to develop CCNILS.

MATERIALS AND METHODS

Plant materials

Forty-five commercial radish varieties: 'Gilcho', 'Daekeun', 'Fall beauty', 'Celadon', 'Samyang', 'Jangsu', 'Seokwangdabal', 'Seoho', 'Yeongdong', 'Seonggong', 'Sweet home', 'Jangseong', 'Summer best', 'Hwangto kimjang', 'Cheongjinju', 'Cheonghak', 'Sunny green top', 'Hacheong' from Nongwoo Bio Co., Ltd. 'Dancheong', 'Cheongun', 'Taebaek', 'Gwandong yeorum', 'Daeburyeong yeorum', 'Baekja', 'Cheongbok', 'Taewang', 'Taepyong', 'Haryong', 'Daehyeong chuseok' from Seminis Korea Inc. 'Green beauty', 'Youngsan', 'Togwang', 'Hachu', 'Autumn winner', 'Summer winner' from Dongbu Hannong Inc. 'Myeongsan', 'Baekgyeong', 'Yeonggwang', 'Jangwon', 'Summer green', 'Taecheong', 'King kong' from Syngenta Korea Co. 'Chamisl', 'Hangaeul' from Sakata Korea Co., Lit. and 'Pyeongji yeoreum' from the National Horticultural Research Institute were used to identify and classify the *S*-haplotypes by PCR-RFLP in order to select parental materials possessing different *S*-haplotypes.

Marker Assisted Selection (MAS) procedure

MAS was performed in the F_2 generation by PCR with primer pairs of RPS5 + RPS15 or SRK3 + SRK5 for the class-I *S*-haplotypes and RPS3 + RPS21 for the class-II *S*-haplotypes (Lim *et al*. 2002).

The total volume of PCR was 25 µL with 25 pmol of each primer, 100 pg of template DNA, 200 µM of each dNTP, one unit of DNA polymerase (Boehringer Mannheim, Germany), and a reaction buffer containing 250 mM KCL, 100 mM Tris-HCl at pH 8.85, 50 mM $(NH_4)_2SO_4$, and 20 mM $MgSO_4$. The PCR conditions were pre-denaturation for 5 min. at 94°C, 42 cycles of 1 min. at 94°C, 2 min. at 55°C, and 3 min. at 72°C, and an extension of 10 min. at 72°C with a thermal cycler (i-Cycler, Bio-Rad, Inc., Herculus, CA, USA).

The expected *S*-haplotypes of F_1 plants were S_1S_4, $S_{17}S_4$, S_1S_5, and $S_{17}S_5$. Among them, S_1 and S_{17} were class-I haplotypes, and S_4 and S_5 were class-II haplotypes. When one S_1S_4 plant was self-pollinated, the expected segregation pattern of F_2 sib plants was as follows; 1 S_1S_1: 2 S_1S_4: 1 S_4S_4. Thus, when the DNA from one F_2 plant was successfully amplified by both class-I and class-II primer pairs, it was selected as a heterozygous SI plant. In order to ensure the selection of at least one SI heterozygote, four F_2 seeds originated from one F_1 plant were sown. The same method of MAS was performed in each generation.

The amplified fragments were analyzed by electrophoresis on 1.2% agarose gels.

Pollination procedure

For vernalization, seedlings were incubated in a 4°C chamber for six weeks. Each plant was transplanted into a 12-cm plastic pot and moved into a greenhouse set at 15-35°C for flowering and pollination. For self-pollination, any open flowers were removed and approximately ten bud clusters were enclosed with paper bags. When five flowers had opened, the paper bags were briefly removed for pollination. Fifteen young unopened buds 5-8 mm in size were cut in the middle to expose the pistil, and the pistil was fertilized with pollen from the open flowers. After pollination, the flowers were put back in the same paper bag for 2 weeks.

RESULTS

In order to develop numerous SI heterozygous inbred lines in a short time, the SSD method was used. Unlike conventional SSD, where a single seed from each plant is sown without selection (Lee and Yoon 1987), the SI heterozygotes were selected through PCR amplification with a proper combination of primer pairs. The number of seeds to sow should be calculated by considering the cost and selection efficiency. When a heterozygous SI plant is self-pollinated, 50% of the progeny is expected to be heterozygous for the SI trait. When four seeds from each 300 self-pollinated heterozygous SI plants are sown, it is expected that 281.25 heterozygous SI plants could be obtained. The cost and labour of PCR-based selection prevented the sowing of more than four seeds from any one F_1 plant. To find a cost effective DNA extraction method for radish, which contains a lot of polysaccharides (Park *et al*. 2006), three methods were tested. The 'FB plate' method was the most cost efficient (**Table 1**), while the 'DNeasy 96-plant kit' yielded the cleanest bend (Choi *et al*. 2006).

Two sets of parents were selected from summer and autumn varieties, respectively. The parents should have different *S*-haplotypes within populations in order to develop four different SI inbred lines. One set of parents was selected from summer varieties and is referred to as 'population 1' hereafter. The most popular summer variety of 'Gwandong yeoreum' (Seminis Korea, Inc.) with the S_1S_{17} genotype and the disease-tolerant first summer variety of 'Pyeongji yeoreum' (NHRI) with the S_4S_5 genotype were selected as the parents for 'population 1'.The other set of 'Haryeong' (Seminis Korea, Inc.) with the $S_{21}S_{30}$ genotype and 'King kong'

Table 1 The cost of DNA preparation methods in radish.

Method (Company)	Cost[a] ($/sample)	No. of samples/ day/person
'Dneasy 96-plant kit' (Qiagen, CA, USA)	2.62	384
'Wizard magnetic 96 DNA plant system' (Promega, WI, USA)	2.42	384
'FB plate' (Milipore, MA, USA)	0.45	384
CTAB method (Rogers and Bendich 1985)	0.09	30

[a] Cost included two kits, chemicals, 96-well Master block or 1.5 ml microcentrifuge tubes, and 96-well plates.

(Syngenta Korea, Co.) with the S_8S_{26} genotype was selected from autumn radish varieties and is referred to as 'population 2'.

The parents of each population were cross-pollinated and 300 F_1 seeds were sown, respectively. MAS was performed on the F_2 generation, when the SI genotype began to segregate heterozygous and homozygous plants. PCR amplification with RPS5 + RPS15 and RPS3 + RPS21 was performed on 1,168 F_2 plants of 'population 1'. In 'population 2', the MAS was performed on 1,164 F_2 plants with the RPS3 + RPS21 and SRK3 + SRK5 primers (Lim *et al.* 2002).

272 and 175 SI heterozygous F_2 plants were selected from 'population 1' and 'population 2', respectively. In 'population 2', the class-specific primers could not distinguish between the S_8S_{30} genotype belonging to the class-I/class-I haplotype and the $S_{21}S_{26}$ genotype belonging to the class-II/class-II haplotype. Thus, approximately half of the SI-heterozygote lines were missed in the F_2 generation.

The selected plants were self-pollinated, and 346 F_2 plants bore more than four seeds. After PCR amplification with the proper primer pairs, 192 and 135 SI-heterozygote F_3 plants were selected from each population, respectively. The selected F_3 plants were self-pollinated, and 187 and 131 SI-heterozygote F_4 plants produced more than four seeds from each population, respectively (**Table 2**). Following self-pollination, 127 and 112 SI heterozygote F_5 plants were selected, respectively.

The seed-bearing ability of some F_3 lines were investigated by cross-pollination between SI homozygous inbred lines, which were derived from one heterozygous SI plant. Results showed that the NILs derived from '114' heterozygous SI plants produced 4.86 seeds per flower pollination. Other NILs produced 0.11-3.27 seeds per cross-pollination (**Table 3**). Compared to the average seed number of self-pollination in inbred lines of radish, 1.5, the NILs derived from '114' produced more seeds (Lee 1979). From the seed-bearing test with flower pollination, it became clear that the parental seeds for double-crosses can be produced by bee pollination using CCNILs derived from an inbred line possessing different *S*-haplotypes.

Table 2 The size of generations selected with PCR-based markers on each population to make heterozygous SI lines using SSD in radish.

Generation	Steps	№ of lines (plants)		Total
		Population 1	Population 2	
F_1	Sowing	300	300	600
	Seed bearing	292	291	583
F_2	Sowing[a]	292 (1,168)	291 (1,164)	583 (2,332)
	Selection[b]	272 (272)	175 (175)	447 (447)
	Heterozygous	272 (1,088)	175 (700)	447 (1,788)
	Homozygous	11	18	29 (116)
	Failure[c]	9	98	107
	Seed bearing	205 (205)	141 (141)	346 (346)
F_3	Sowing[a]	205 (820)	141 (564)	346 (1,384)
	Selection[b]	192 (192)	135 (135)	327 (327)
	Heterozygous	192	135	327
	Homozygous	7	6	13
	Failure	6	0	0
	Seed bearing	187 (187)	131 (131)	318 (318)

[a] Four seeds were sown from each F_1/ F_2 plants to select heterozygous SI genotype.
[b] Selection was done through PCR amplification with primer pairs of RPS5 + RPS15 and RPS3 + RPS21 for the 'population 1' and SRK5 +SRK15 and RPS3 + RPS21 for the 'population 2'.
[c] Failure was severe in the 'population 2'at the F_2 generation, because class-specific primers could not distinguish between the S_8S_{30} and the $S_{21}S_{26}$ genotypes.

Table 3 Number of seeds per flower cross-pollination between two CCNILs with different homozygous *S*-haplotypes from the 'population 1'.

Seed parent (Class of *S*-haplotype)	Pollen parent (Class of *S*-haplotype)	№ of seeds/ Flower pollination[d]
19-a (I)[a]	19-b (II)	0.11
19-b (II)[a]	19-a (I)	3.27
114-a (I)[b]	114-b (II)	4.86
114-b (II)[b]	114-a (I)	2.67
86-a (I)[c]	86-b (II)	0.75
86-b (II)[c]	86-a (I)	1.83
Mean		1.93

[a]19-a and 19-b were derived from a A19 SI heterozygous F_3 plants.
[b]114-a and 114-b were derived from a A114 SI heterozygous F_3 plants.
[c]86-a and 86-b were derived from a A86 SI heterozygous F_3 plants.
[d]Flower pollination was done using flowers considered to be opened the day of cross-pollination in a paper bag. Ten flowers were cross-pollinated.

DISCUSSION

Self-incompatibility has been incorporated into the hybrid breeding of *Brassica* vegetables. Producing uniform hybrid seeds is a major step, and propagating parental lines is also a crucial step. For the production of uniform hybrid seed, inbred lines possessing high SI activity have been used as parents by commercial seed companies in Korea. For the propagation of parental lines, a high concentration CO_2 treatment has been used to avoid SI (Nakanishi *et al.* 1969; Park and Jung 1983; Horisaki *et al.* 2003). Since the CO_2 treatment has made it possible to produce a considerable amount of seeds from inbred lines, it has been widely used for developing single-cross hybrid varieties in *Brassica* vegetables.

Double-crossing (DC), in which hybrid varieties are used as parents, was suggested to overcome SI for parental seed production, and it was utilized for hybrid seed production (Nieuwhop 1968; Niikura and Matsuura 1998). Using similar inbred lines possessing heterozygous SI, Nieuwhop (1968) tried to increase the uniformity of hybrid seeds in *Brassica* vegetables. However, selecting interesting *S*-haplotypes was nearly impossible with conventional crossing methods. The conventional method requires crossing with many tester lines and investigating the progenies to make selections. Thus, phenotypically similar cross-compatible inbred lines were used as parents for making DC varieties in radish.

The mean seed number per pod of radish was 3.4 and that of Chinese cabbage was 6.5 using collected germplasm (unpublished data). Thus radish breeders have been trying to develop DC varieties to enhance parental seed production. However, the uniformity of double-crossed hybrid seed is low because the genetic bases of the parental lines for F_1 hybrid production are heterogeneous.

Highly uniform DC hybrid varieties can be developed using cross-compatible near-isogenic lines (CCNILs) in which only *S*-haplotypes are different. In this study, we developed 318 F_4 plants of heterozygotes in the *S*-locus through MAS (**Table 2**). The horticultural traits were investigated to check the uniformity of each line. Fifty F_4 lines were sown in a plastic house on Nov. 2, 2004. During three months of cultivation, the temperature was controlled at about 10°C. Each line showed different horticultural traits between lines but showed uniformity within lines (**Fig. 1**). The self-pollinated seeds of these inbreds can be divided into one set of CCNILs by MAS.

Out-crossing crops show inbreeding depression following several rounds of self-pollination (Bhalla 1999). Even though CCNILs possess different *S*-haplotypes, breeders worry that about 4~6 rounds of self-pollination will cause inbreeding depression in seed production. The seed productivity of 3 CCNILs was tested using flower pollination with inbreds of F_4 plants derived from 3 different F_3 plants. The seed number per flower pollination varied from 0.11 to 4.86. Considering that he average seed number per bud pollination is 1.5, which was carried to avoid SI, an average seed number per flower pollination of 1.95 shows the possibility of using CCNILs in breeding (**Table 3**).

We developed CCNILs in short period of time using SSD and MAS (Lee and Yoon 1987; Nishio *et al.* 1994; Lim *et al.* 2002; Park *et al.* 2006).

When F_1 hybrid seeds are produced with insect pollination, it is important to synchronize the flowering time by adjusting periods of vernalization, transplanting, and so forth. If the flowering duration is different, the optimal den-

Fig. 1 Uniform plant shapes of four inbred lines planted in a vinyl house at NHRI. (**A**) An inbred line with high plant vigor and sinuate leaf blades; (**B**) An inbred line of medium plant vigor, semi-erect leaves and obovate leaf blades; (**C**) An early bolting inbred line; (**D**) an inbred line of low plant vigor and horizontal leaves.

sity of the parental lines should be determined to maximize usable flowering time as long as possible. The form of floral organs should be considered because bees tend to gather easily around available flowers. Otherwise, this tendency may cause uneven seed bearing (Horisaki *et al*. 2003). When we use CCNILs for parental seed production, the synchronization of flowering time is not required because the CCNILs are genetically identical. The CCNILs can be used for developing highly uniform DC varieties but also as a highly pure single-crossed variety, if the CCNILs show good horticultural traits. We found tens of male-sterile (MS) inbreds during developing CCNILs. Those CCNILs can be used as maintainers of MS when they show good combination ability between other inbreds. In total, using CCNIL for breeding double and single crossed varieties has many practical advantages in radish.

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