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ORIGINAL PAPER

Developing stable progenies of ×*Brassicoraphanus*, an intergeneric allopolyploid between *Brassica rapa* and *Raphanus sativus*, through induced mutation using microspore culture

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Abstract Induced mutations were used to improve the low seed fertility of an intergeneric allopolyploid, 'Baemoochae,' ×*Brassicoraphanus*, synthesized following hybridization between *Brassica rapa* and *Raphanus sativus*. The mutagen *N*-methyl-*N*-nitroso-urethane (NMU) was added to microspore cultures. Four lines of nine in the Mi_2 generation showed very high fertility under controlled pollination. The progeny lines (Mi_3) confirmed this result under open pollination, and excellent uniformity was observed in plants grown in the field, as well as in their AFLP profile. On attaining high fertility and uniformity, one of the lines was released to farmers as a new leafy vegetable crop. The original nine lines shared very similar AFLP banding patterns, without any large differences between the high and low seed fertility lines. Thus, mutation induction accelerated genetic stabilization of a newly synthesized allopolyploid, ×*Brassicoraphanus*.

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Introduction

Brassica species are sources of edible oil, vegetables, fodder, and condiments in the human diet. The three basic genomes are referred to as aa for *B. rapa*, bb for *B. nigra*, and cc for *B. oleracea*; three allopolyploid genomes originated following natural hybridizations between these basic genomes and are designated aabb for *B. juncea*, aacc for *B. napus*, and bbcc for *B. carinata* (Gómez-Campo and Prakash 1999). Besides these *Brassica* species, *Raphanus sativus* (rr) (radish) is an important vegetable worldwide, and various related wild species have attracted research attention as potential germplasms for improvement of *Brassica* crops (Warwick and Black 1991). Since the syntheses of intergeneric hybrids between *R. sativus* and *B. oleracea* by Sageret (1826, cited by Prakash et al. 2009) and Karpechenko (1927) and the triangle theory of U (1935), numerous reports of hybridizations, not only interspecific but also intergeneric and even intertribal, have been documented in Brassicaceae (Namai et al. 1980; Olsson and Ellerström 1980; Prakash et al. 2009).

Specific traits have been introduced across intergeneric barriers. Some notable hybridization achievements include the introduction of beet cyst nematode resistance from *R. sativus* to *B. rapa* (Dolstra 1982) and to *B. napus* (Lelivelt et al. 1993; Peterka et al. 2004), alternaria leaf spot resistance from *Sinapis alba* to *B. napus* (Primard et al. 1988), and male sterility-inducing cytoplasm from *Raphanus* and other wild species to crop species (Bannerot et al. 1974; Prakash 2001); the creation of a novel sterile male by fusion of two different nuclear genomes in a parental cytoplasm (Bang et al. 2002); and the alteration of oil quality by developing a hybrid of *B. napus* and *Orychophragmus* (Ma and Li 2007). However, attaining high fertility is always a constraint in allopolyploid breeding (Prakash et al. 2009).

Radicole, an alloploid hybrid between radish (*R. sativus*) and cole crop-cabbage (*B. oleracea*), was released as a forage crop, but its fertility was very poor with only two to three seeds per siliqua resulting in 420 kg/ha (McNaughton 1979). Another crop, raparadish, was synthesized by hybridizing *B. rapa* and *R. sativus* and released as an intercropping crop for beet cyst nematode protection; however, raparadish also had reduced fertility with only two to three seeds per siliqua (Dolstra 1982; Lange et al. 1989).

We previously reported the development of a new vegetable crop 'Baemoochae' (a name combining the first letters of the Korean words *bae*choo for Chinese cabbage, *moo* for radish, and *chae*so for vegetable, in arbitrary order), ×*Brassicoraphanus*. This crop was obtained following hybridization between *B. rapa* ssp. *pekinensis* (big head Chinese cabbage) and *R. sativus* (big root radish) (Lee et al. 1989, 2002). It also exhibited low seed fertility, as low as two to three seeds per siliqua. Chromosome cytology using genomic in situ hybridization (GISH) confirmed that this crop is a true allotetraploid carrying 38 chromosomes in its genome composed of full chromosome complements of *Brassica* and *Raphanus* (Lim 2001). Twelve functional organic compounds including β-sitosterol were also identified in 'Baemoochae,' of which some were from *Brassica* and some from *Raphanus* (Rhee et al. 2007). Recently, highly fertile populations of ×*Brassicoraphanus* (synthesized from another combination *R. sativus* × *Brassica alboglabra*) were developed by mass selection carried out to the F_{10} generation (Chen and Wu 2008).

Induction of mutation during microspore culture was attempted to improve the seed fertility and the uniformity of 'Baemoochae,' ×*Brassicoraphanus*. The embryogenesis in the microspore culture of this crop has already been described (Hong and Lee 1995). Previous studies have shown that fixed mutants can be obtained by induced mutation during microspore culture (Swanson et al. 1989; Barro et al. 2001; McClinchey and Kott 2008). Treatment with the mutagen *N*-methyl-*N*-nitroso-urethane (NMU) during microspore culture was also well established in broccoli (Jeong and Lee 1997). In this study, a number of inbred lines with high fertility and high uniformity in successive generations were developed by treatment with NMU during microspore culture.

Materials and methods

Plant materials

Progeny lines of the original plant (line code: OV115C-4) and some F_1 crosses between them of the synthetic intergeneric allopolyploid 'Baemoochae,' ×*Brassicoraphanus* were grown for trait observation in the field during the fall season every year from 1993 onward. Several adult plants of each line were routinely transplanted to 30-cm-diameter pots to produce seeds of the next generation; these plants were kept at 5–10°C for vernalization in a protected polyethylene film house for about 30 days and induced to flower at 12–30°C in the same greenhouse during the winter-spring season. An F_1 cross between BB#4, which was derived from an anther culture of OV 115C-4 and a pedigree line of OV-115C-4, was used for mutagenesis in this study. The check BB#1 was a derived line from microspore culture of BB#4. All of the materials in this trial were maintained for more than ten generations. The Chinese cabbage, one of two parents of 'Baemoochae,' ×*Brassicoraphanus*, employed for AFLP analysis was a similar cultivar chosen carefully from commercial F_1 hybrids because the same F_1 from 1990 was not available; the radish was the identical F_1 hybrid, 'Taeback.'

Mutagenesis in the microspore culture

The basic protocols from bud collection to the incubation of microspores at 25°C in the dark for culture were as previously reported (Hong and Lee 1995). The effects on embryo induction of 0.1 mg/L of benzyl adenine, 0.01 and 0.1 μM of NMU in the culture medium, and NLN 13 (Duchefa Biochemie BV, Haarlem, The Netherlands), followed by 72–96 h heat shock at 32.5°C were examined with the above-mentioned plant materials. Trials were repeated more than a dozen times because the embryo yield was generally low, from 0 to less than 10 per Petri dish. The NMU treatments of 0.01 and 0.1 μM were as described previously for broccoli mutagenesis during microspore culture (Jeong and Lee 1997). For plant regeneration, embryos were transplanted to MS medium (Murashige and Skoog 1968) supplemented with 2% sucrose, which enhanced embryo yield (Supplementary Table 1). Normal plants with well-developed shoots and roots were transplanted to a soil pot for acclimatization, and abnormal plants were subcultured in the MS medium supplemented with 1.0 mg/L of BA and 0.2 mg/L of NAA (Keller and Amstrong 1977) to induce normal growth. Acclimatized plants were vernalized in a low temperature greenhouse and grown routinely at 12–30°C for seed production.

Fertility and uniformity test

In total, 24 acclimatized plants were grown in the greenhouse during the winter-spring season. Only ten Mi_1 (first generation derived from the microspore culture with or without mutagen) plants developed normally and produced pollen, and self-pollinations were attempted (Supplementary Table 2). The self-pollination in this study was carried out on 5 flowers and 15 buds on the same branch. More than

three branches of each plant were pollinated. The Mi_2 seeds of every Mi_1 plant, except one coded 554, were grown in the field during the fall season. Two to three adult plants in each line were transplanted to pots for seed production of the Mi_3 generation. Four of the nine lines had exceptionally high seed fertility and their Mi_3 lines showed excellent uniformity in the field. Twenty to 40 plants of the four Mi_3 lines were grown in the same field with two replications to confirm their fertility in open pollination. Seeds from 100 pods collected randomly from each line were counted for fertility. Seed yield was also measured by counting the total seeds harvested from all plants of the two replications.

After confirmation of sustainable high fertility and high uniformity, bolting sensitivity was tested with two check lines in a randomized complete block design by direct sowing on April 12.

AFLP analysis

DNA samples were collected from each of 20–40 plants of the four mutants with high seed fertility (Mi_3) and from two check lines to confirm the uniformity of polymorphic bands in the AFLP profile. Nine lines of the Mi_3 generation and two check lines were also subjected to AFLP analysis to identify their similarity or diversity and the difference in AFLP band patterns between the high and low seed fertility lines. DNA samples were collected from four plants randomly from uniform lines and from 7 to 10 plants selected for morphological differences in the field. AFLP analysis followed the procedure of Vos et al. (1995) with some modification. Genomic DNA was extracted from the samples and mixed in equal amounts from each plant of interest just before the experiment. Purified genomic DNA was digested with *Eco*R1 and *Mse*1 and adapters were ligated to the restriction fragment ends. Pre- and selective amplifications of the ligated DNA were conducted with ten primers designed previously for Chinese cabbage. The PCR products were loaded on 6% polyacrylamide gels and separated. The DNA bands with 80–800 bp visualized by silver staining were scored. The similarity of the lines was estimated by Nei's distance equation, and cluster analysis was performed with the unweighted pair-group method (UPGMA) using NTSYS-pc.

Results

Only ten plants (eight treated and two non-treated with 0.01 μM NMU) produced pollen and seeds among the 24 plants transplanted after acclimatization that had been regenerated from 116 embryos. However, the seed fertility of the ten seed-producing plants was very low and varied by pollinated flower stalk, except for the plant coded 558 which showed quite high seed fertility at 4.9 seeds per pollination (Supplementary Table 3. Table 1). In the next generation (Mi_2), the plants had varying fertility similar to the Mi_1 plants (Supplementary Table 4). The pedigree lines of the four plants coded 548, 551, 560, and 562 displayed exceptionally high fertility on average and in their best flower stalks (Table 1). This was the first such high fertility to be observed since synthesis in 1985. However, the line coded 558, which was descended from the most fertile plant in the Mi_1 generation, was not included in this high fertility group. In the fall season, plants were grown in two replications with 30 plants each in the field. The plants resembled each other (Fig. 1a) and were uniform in appearance. The upper part of the plant was similar to that of the wild line, with only slight differences in plant spreading, although the root was quite different, slender like a carrot root and not plumped as in the parent line (Fig. 1b).

In the following year, seed fertility was confirmed by open pollination in the field, while growing together with two control lines, BB#1 and BB#4. Fertility, evaluated by the number of seeds per 100 pods, was more than 7.6 seeds/siliqua and yield was more than 37.5 g/plant, which was equivalent to more than 750 kg/ha. In comparison, the control lines produced 2.1–2.3 seeds/siliqua and 20.4–21.0 g/plant (Supplementary Table 5 and Table 2). Differences in seed yield between the control and mutants resulted from the abortion rate of seeds per siliqua. Almost all the ovules were mature in the mutant plant (line code 551), whereas

Table 1 Number of seeds per siliqua (seed fertility) on artificial self pollination of Mi_1 plants and Mi_2 lines derived from microspore culture with or without NMU treatment in 'Baemoochae', ×*Brassicoraphanus*

Plant code	NMU treat. (0.01 μm)	Number of seeds per siliqua			
		Mi_1 plants		Mi_2 lines	
		Mean[a]	Best stalk[b]	Mean[c]	Best stalk[d]
548	Yes	0.1	0.8	1.2	7.5
550	Yes	0.4	0.6	0.6	3.8
551	Yes	0.2	0.4	1.2	6.8
552	Yes	0.1	0.9	0.2	1.0
554	No	0.1	0.3	–	–
556	No	0.3	1.6	0.0	0.3
558	Yes	0.3	4.9	0.5	1.6
559	Yes	0.3	1.0	0.5	2.6
560	Yes	0.5	3.0	1.4	8.6
562	Yes	0.2	0.5	1.8	6.6
289	No (wild)	0.2	0.7	0.5	1.4

[a] Mean of 27–414 pollinations

[b] Best of 3–21 stalks

[c] Mean of 90–929 pollinations

[d] Best of 7–39 stalks

Fig. 1 Morphology of roots of four mutant lines (**a**), of representative plant of the wild line and a mutant (**b**), ovules matured and degenerated in the wild and a mutant, line code 551 (**c**) in 'Baemoochae', ×*Brassicoraphanus*

Table 2 Number of seeds per siliqua and seed yield on open pollination and polymorphism AFLP profile of four mutant lines in Mi_3 generation of 'Baemoochae' ×*Brassicoraphanus*

Line code	No. of plants	Number of seeds per siliqua	Seed yield (Mi_4)			Ratio of polymorphic bands in AFLP (%)
			Harvested (g)	Per plant (g)	Per ha (kg)	
548	30	7.6	1.253	41.8	835	4.2
551	40	8.8	2.296	57.4	1148	4.9
560	40	7.6	1.967	49.2	983	4.3
562	40	8.0	1.498	37.5	749	4.0
BB#1 (check A)	39	2.3	559	20.4	410	4.5
BB#4 (check B)	21	2.1	441	21.0	421	5.4

Number of seeds per siliqua was counted from 100 siliqua. Seed yield per ha is 20,000 times of the seed yield per plant

only three ovules were mature and the others degenerated before maturation in the control line BB#1 (Fig. 1c).

The polymorphism displayed in the AFLP analysis for uniformity verification was 4.0–4.9% from a total of 672–919 bands in the high uniform lines and 4.5 and 5.4% in the checks, BB#1 and BB#4, respectively (Supplementary Table 6 and Table 2). Polymorphism of less than 5.0% can be considered very uniform, since a doubled haploid line of Chinese cabbage displayed 4.0% polymorphisms (unpublished data).

Another AFLP analysis with the same ten primers was carried out with 13 samples consisting of nine Mi_3 lines, two wild check lines, and the two parents of 'Baemoochae,' ×*Brassicoraphanus*, Chinese cabbage and radish, to confirm their similarity and band patterns. A dendrogram with a total of 680 bands that originated from 13 and 15 polymorphic bands revealed that the 'Baemoochae,' ×*Brassicoraphanus* lines were more remote in genetic distance from radish, the male parent, than from Chinese cabbage, the female parent. The 'Baemoochae,' ×*Brassicoraphanus* lines were much closer to each other except for the line coded 552. This line was considered to be a negative mutation compared to other lines. The lines originating from microspores treated with NMU were very similar to each other without any difference between high and low fertility lines, and slightly distant from the non-treated lines and the two check lines (Fig. 2).

The band patterns of each line displayed by AFLP were compared with the fertility of their progenitor Mi_2

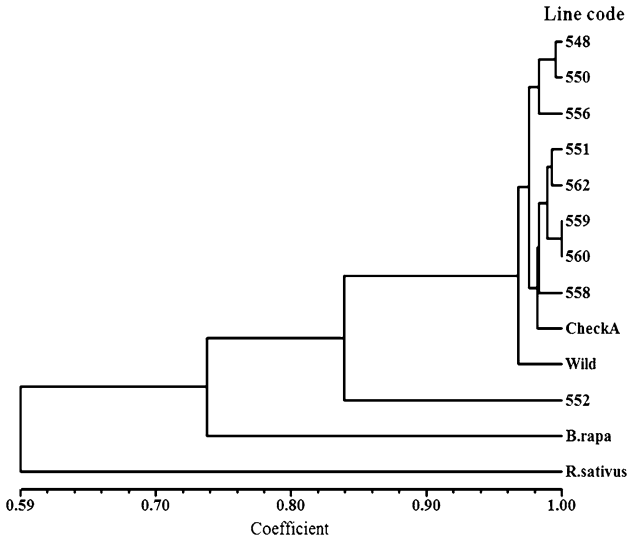

Fig. 2 Dendrogram of nine lines of Mi_3 generation and one check and one wild lines in 'Baemoochae', ×*Brassicoraphanus* and two parents, *Brassica rapa* and *Raphanus sativus*-based NTSYS program from AFLP

(Table 3). In total, 'Baemoochae,' ×*Brassicoraphanus* showed 676 polymorphic bands, consisting of around 47% common, 29% female, 19% male, and 5% novel bands; in addition, approximately 9.5% of 478 bands of female Chinese cabbage and 16.5% of 446 bands of male radish were missing, with no large difference between high and low fertility, high and wild, and high and check lines. No distinct band pattern was observed in the tested lines, even

Table 3 Relationship between the number of seeds per siliqua at Mi_2 and the rate of maintained and lost bands at Mi_3 detected with AFLP analysis in Baemoochae, ×*Brassicoraphanus*

Line code	Number of seeds per siliqua at Mi_2[a]	Maintained band (%)				Lost band (%)	
		Common of parents	Female parent (*B. rapa*)	Male parent (*R.sativus*)	Novel	Female parent (*B. rapa*)	Male parent (*R.sativus*)
560	8.6	46.3	29.5	18.9	5.3	9.2	16.4
548	7.5	47.1	29.5	18.0	5.5	9.2	17.3
551	6.8	46.8	29.1	19.0	4.7	8.8	16.1
562	6.6	46.6	29.7	19.1	4.6	9.6	16.8
550	3.8	47.2	29.4	17.9	5.5	9.6	17.5
559	2.6	46.5	29.5	18.8	5.1	9.2	16.6
558	1.6	46.4	29.6	18.9	5.1	9.2	16.4
552	1.0	46.6	29.5	19.3	4.6	9.8	16.4
BB#4 (check B)	1.0	46.5	29.4	19.5	4.6	9.8	15.9
Wild	0.7	47.1	29.4	18.9	4.6	10.3	17.0
556	0.3	46.8	29.6	18.4	5.3	8.8	16.5

Total of polymorphic bands: ×*Brassicoraphanus* lines:676; total bands of *B. rapa band*: 478 (70.7%), total bands of *R. sativus bands*: 446 (66.0%)

[a] Number of seeds per siliqua: result of controlled pollination during the winter-spring season

Table 4 Characteristics of high fertile mutants compared to check lines in spring growing

Line code (BB series name)	Uniformity	Plant wt.(kg)	Leaf wt.(kg)	No. of leaves (ea)	Leaf length (cm)	Flower stock length (cm)
Check A (BB#1)	Fair	1.5	1.4	17.0	46.7	Uneven
Check B (BB#4)	Fair	1.4	1.3	14.7	47.0	23
548 (mutant BB#11)	Excellent	1.5	1.4	17.7	40.0	13
551 (mutant BB#12)	Excellent	1.3	1.1	14.0	40.3	17
560 (mutant BB#13)	Excellent	1.6	1.5	19.0	39.7	14
F value		2.02	0.99	2.78*	3.27**	–

in the line coded 552, which was distant from the other lines in the dendrogram, and no relationship was revealed between the fertility level and the band patterns. However, all of the lines, including the check lines, maintained about 10% more female bands than male bands, lost about 6–8% less female bands than male bands, and exhibited novel bands of about 5% in total. These visual facts suggest that more genetic elements of the male parent were lost than those of the female parent during the generation progress, and no dramatic gain or loss of genetic factors arose from the low concentration treatment of NMU.

Several new findings were determined during generation of the mutant lines. A high concentration of sulforaphene (Lim et al. 2009), a functional substance for anticancer and anti-super bacteria, was recognized (Korean patent no. 10-878699-0000, 10-2008-62828). The plants also showed a stable yield in the fall (around 2.2 kg/adult plant) with high fertility and uniformity. No particular feature was demonstrated except less growth of the longest leaf than in the control lines during the spring growing season (Table 4). Thus, line BB#12 (line code 551), among the four mutants which could not be distinguished from each other, was released as a leafy vegetable and registered for variety protection in Korea.

Discussion

Occurrence of low seed fertility is a very common phenomenon observed in synthetic allopolyploids in *Brassica* coenospecies (Prakash 2001; Prakash et al. 2009). Two hypotheses, the meiotic irregularity of chromosomes (Richharia 1937; Howard 1938) and genomic imbalance rather than cytological irregularity (McNaughton 1979; Dolstra 1982; Tokumas and Kato 1988), were proposed to explain the low fertility in the *Raphanobrassica*. DNA sequence elimination and gene expression alteration were recently suggested as causes of meiotic irregularity of chromosomes and genomic imbalance following the instability of allopolyploids (Lukens et al. 2006; Chen and Wu 2008; Gaeta et al. 2007, 2009; Xu et al. 2009). The initial plant of

'Baemoochae,' ×*Brassicoraphanus* showed several multivalents leading to irregular meiosis (data not shown) and low seed fertility, which had been sustained through more than 15 generations. High fertility was obtained from one of these low fertility materials by induced mutation during microspore culture and stabilized. Chen and Wu (2008) developed a high fertility population in ×*Brassicoraphanus* synthesized from a combination of *R. sativus* × *Brassica alboglabra* by mass selection carried out up to the F_{10} generation. They hypothesized that DNA sequence elimination and/or chromosomal rearrangements occurred on a large scale during the successive selections, which resulted in the loss of enzyme combining and cutting sites and rapid differentiation of the homoeologous chromosomes. Consequently, incompatible factors between both parents could be harmonized in chromosome and gene levels and in turn, diploid-like meiosis was induced. In 'Baemoochae,' ×*Brassicoraphanus*, the nine lines in the Mi_3 generation, consisting of four high fertility lines and five low fertility lines, and two control lines were very close to each other, except for one mutant in the AFLP diagram. Specific DNA fragments of the cytoplasmic parent were less likely to be lost and more remained than from the male parent, as pointed out by Song et al. (1988, 1995); furthermore, the AFLP analysis showed that about 5% novel bands were created, as also noted by Chen and Wu (2008). However, the number of DNA sequences eliminated and created was almost the same in all lines without any major differences between the high fertility and low fertility lines (Table 3 and Supplementary Table 7). This suggests that the elimination and creation of novel DNA sequences were general incidents accompanied by generation advancement in the allopolyploid and not related directly to the high fertility induction in 'Baemoochae,' ×*Brassicoraphanus*. High fertility was induced and stabilized by achieving a diploid-like meiosis regime (Prakash et al. 2009) due to chromosome rearrangements induced by genomic change (Pires et al. 2004; Zao et al. 2007), such as homoeologous chromosome exchange and loss and doubling of homoeologous genes without differential change in the overall expression of homoeologous sets of genes in the allopolyploid (Lukens et al. 2006; Gaeta et al. 2007, 2009). The role of rRNA genes (Chen and Pickard 1997; Ge and Li 2007), referred to as nucleolar dominance, was also proposed for stabilizing the chromosome pairing that resulted in high fertility with preferential stabilization of chromosomes from the rRNA's donor parent (Li and Ge 2007). However, this suggestion remains to be confirmed, since rRNA genes were not detected in the present study. Diploid-like meiosis is controlled by the pairing regulator gene *PrBn*, which restricts the homoeologous pairing in *B. napus* (Jenczewski et al. 2003). Whether such a locus is present in the R genome of the AARR plant also requires further study, as this gene was mapped in the C genome of the AACC plant.

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