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Developing Stable Cultivar through Microspore Mutagenesis in ×*Brassicoraphanus koranhort*, Inter-Generic Allopolyploid between *Brassica rapa* and *Raphanus sativus*

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Abstract

A stable progeny was developed through induced mutation, using microspore culture, of the hybrid (F_1F_1) produced by crossing a newly synthesized, unstable allopolyploid (F_1) and a stable cultivar, BB#1(F_1) in x*Brassicoraphanus*. An F_1F_1 plant was subjected to the induced mutation system established during production of BB#1. Morphological characteristics of the progeny such as color, and leaf number and length, differed from those of BB#1. The bolting time of the progeny in spring cropping was very late compared to BB#1, allowing it to be grown to an adult plant in spring. Genomic *in situ* hybridization analysis of pollen mother cells at prophase I identified 19 bivalents, 10 from *Brassica rapa* and 9 from *Raphanus sativus*. The glucoraphenin content was almost identical to that of BB#1. Two cultivars are available in the baemoochae crop now. These results indicate that induced mutation using microspore culture is a viable method of stabilizing inter-generic allopolyploids between *B. rapa* and *R. sativus*.

Keywords

×*Brassicoraphanus*, Intergeneric Hybrid, Baemoochae, *Brassica rapa*, *Raphanus sativus*

1. Introduction

Following Karpechenko's report in 1927 [1], numerous studies have focused on the allopolyploid, inter-generic hybrid between *Brassica* and *Raphanus*, desig-

nated ×*Brassicoraphanus* [2] [3]. Most ×*Brassicoraphanus* hybrids were unstable; few have been used as breeding material [4] [5] [6]. Production of a stable line from 10 generations of mass selection of ×*Brassicoraphanus* as an inter-generic allopolyploid from *R. sativus* (♀) and *B. oleracea* var. *albogrobula* (♂) was reported [7]. However, the line was not palatable and the oil content was low. Lee *et al.* (2011) [8] reported development of a stable inbred by microspore mutagenesis, using n-nitroso n-methyl urethane (NMU), applied to an unstable line, BB#4 of ×*Brassicoraphanus*, an allopolyploid from *B. rapa* (♀) and *R. sativus* (♂). This new inbred had high nutritional value and sulforaphene content, which has marked anticancer and antibacterial properties [9]. The inbred was very tender and crispy in texture, and sweet and pungent in taste [10]. The inbred was given the crop name "baemoochae" and released with the cultivar name BB#1 after registration for variety protection in 2009.

There has been interest in developing another stable line from different parental germplasm, thereby expanding the industrial use of baemoochae and confirming the effectiveness of microspore mutagenesis for speciation of allopolyploids from the cross-pollination of *B. rapa* and *R. sativus*. When a stable accession was recognized, it was named as BB#5 for conveniences in future's affaires, such as the cytological study, the analysis of content of glocosinolate and registration for variety protection.

2. Materials and Methods

2.1. Developing Stable Inbred

A unique landrace of kimchi cabbage, jombaechoo (*B. rapa*), and an accession from collections of the landrace gaetmoo (*R. sativus*), were used as parents to develop a new, stable baemoochae line. They were supplied by The Center of Germplasm, The Rural Development Administration in Korea with the introduction no. of PI204163 for *B. rapa* and IT216097 for *R. sativus*. They were known to be maintained high content of disulfide constituent of sulforaphene in an analysis. Jombaechoo is much similar to radish in the leaf morphology, an enlarged terminal lobe and smaller lateral lobes. Gaetmoo is a typical wild type in the root shape with numerous thin roots from the slender and little bit small root. Jombaechoo and gaetmoo were self-pollinated two and three times, respectively, to produce more uniform lines. Bud pollination was performed on jombaechoo pistils. Anthers from jombaechoo were removed 1 day before being pollinated by gaetmoo pollen [11]. Ten-day-old immature hybrid ovules were cultured in darkness at 25˚C on modified B_5 medium (Duchefa Co.) [12] supplemented with 600 mg/L $CaCl_2 2H_2O$, 800 mg/L glutamine, 100 mg/L L-serine, 0.1 mg/L NAA, 0.1 mg/L BAP, 0.1 mg/L 2,4-D, and 30 g/L sucrose. The ovule-culture derived plants were transplanted to soil for acclimatization. A 0.3% colchicine solution was applied to the growing points of established plants for 3 days, using absorbent cotton, to double chromosomes. The size and wave edge of leaves were different to both parents and flowers were white in color, as if the inter-generic hybrid. The F_1 plants capable of producing pollen were self-polli-

nated and cross-pollinated reciprocally to BB#1. Three to five fresh flowers and approximately 10 buds on the same flower branch were self-pollinated, and non-pollinated buds were removed. This procedure was repeated on several branches to acquire sufficient seed. Cross pollination to BB#1 was managed in the same way as the self pollination with flowers. The plant 07OV-127 was subjected to microspore mutagenesis. Liquid NLN 13 medium [13], supplemented with 0.1 mg/L BA, was added to the microspore culture. A stock solution of n-nitroso n-methyl urethane (NMU) was added to the culture solution containing a microspore density of 10^4 per mL to a final concentration of 0.01 μM. Embryos derived from microspores were transplanted to MS_2 medium supplemented with 2% sucrose to MS medium for plant regeneration [14]. Normal plants with well-developed shoots and roots were transplanted to pots for acclimatization. Abnormal plants were cut into pieces of about 5.0 × 5.0 mm and sub-cultured in MSk medium (1.0 mg/L BA, 0.2 mg/L NAA and 2% sucrose added to basic MS medium) to induce normal plants [14]. Acclimated plants were vernalized in a low-temperature greenhouse, in which the temperature was about 2˚C to 7˚C in the night time and 10˚C to 12˚C in the day time for 25 days, without colchicine treatment for chromosome doubling, followed by exposure to 12˚C - 30˚C for seed production.

Data collected from pollinations in earlier generations were abbreviated for shortening the volume of the manuscript and stated in the text for understand the flow of the story.

2.2. Cytology

Meiotic chromosome behavior of BB#5 from prophase I to anaphase II was observed as described by [15] [16], with minor modifications. An unstable line, BB#4, and the stable line BB#1 were compared. Chromosome abnormalities were scored and analyzed in 500 pollen mother cells (PMCs) using a fluorescence microscope (Olympus BX53, Japan) equipped with a CCD camera (Leica DFC365 FS, Germany).

Genomic *in situ* hybridization (GISH) analysis, using blocking DNA of *R. sativus* and probe DNA of *B. rapa*, was applied to BB#5 to determine its meiotic chromosomal constitution [17]. The CTAB-extraction method was used to extract and purify total genomic DNA from young leaves of *B. rapa* and *R. sativus* [18]. Genomic DNA from *B. rapa* was labeled with dig-11-dUTP (Roche, Germany) by nick translation and used as a probe, while the unlabeled DNA of *R. sativus* was fragmented at 100˚C for 7 min and used as a block. During GISH analysis, the slides were pretreated with RNase A buffer (100 μg·ml $^{-1}$ RNase A in 2× SSC) at 37˚C for 1 h and then treated with 100 μg/ml pepsin (Sigma, USA) in 0.01 M HCl at 37˚C for 10 min. After washing for 5 min with 2× SSC, the slides were post-fixed with 4% paraformaldehyde in 2× SSC, washed with 2× SSC, and dehydrated in an ethanol series (70, 90, and 100%) for 2 min each. A hybridization mixture (50% formamide, 10% dextransulfate, 2× SSC, 5 ng·μl^{-1} unlabeled genomic DNA, and 50 ng·μl $^{-1}$ of labeled genomic DNA as a probe) was pre-

pared and then adjusted to 40 μl per slide with nuclease-free water. The GISH mixture was denatured at 90˚C for 10 min and immediately stored on ice for at least 5 min prior to mounting on slides. Chromosomes were then co-denatured at 80˚C for 3 min on a ThermoBrite (Fisher Scientific, USA), then hybridized overnight at 37˚C. The slides were washed in 2× SSC for 30 min at RT, 0.1× SSC for 30 min at room temperature and finally in 2× SSC for 15 min at 42˚C. A dig-labeled probe was detected using an anti-dig-FITC conjugate (Sigma, USA) at 37˚C for 1 h. The slides were dehydrated in an ethanol series (70, 90, and 100 %) for 3 min each, air-dried, and counterstained with 1 μg/ml DAPI (Roche, Germany) in Vectashield (Vector Lab., Inc., USA). Images were captured with an Olympus BX53 fluorescence microscope equipped with a Leica DFC365 FS CCD camera, and processed using Cytovision version 7.2 (Leica Microsystems, Germany) software. Further image enhancements were performed using Adobe Photoshop CC.

2.3. Glucosinolate Analysis

Seed contents of total glucosinolate and glucoraphenin of BB#5, which is the precursor of sulforaphene, were measured according to the optimized official method, ISO 9167-1(1992) [19]. Two hundred milligrams of dried seeds were mixed with 10-ml boiled 70% methanol and incubated at 70˚C for 20 min to inactivate plant tissue enzymes. Following centrifugation at 3000 g for 15 min at 4˚C, the supernatant was concentrated to 3 ml under N_2 gas. Residue was resuspended with 1 mL of 20 mM sodium acetate buffer and loaded onto a DEAE Sephadex-A25 column. Purified sulfatase (75 μL, 150 U/mL) was also loaded onto a Sephadex-A25 column and stored at 37˚C for 12 h. Desulfo-glucosinolates were eluted 1.5mL ddH_2O (HPLC grade). The total eluted solution was concentrated to 0.5 mL for HPLC analysis. The sample was filtered through a 0.45-μm PVDF syringe filter before injection. The samples were analyzed on Varian LC-900 and separated on C-18 reverse phase column (Zorbax Eclipase XDB-C18, 4.6 × 250-mm i.d., 5-μm particle size, Macherey-nagel, Germany) at column temperature of 35˚C. Compounds were detected by UV detector set at 229 nm and separated by following program with a water-acetonitrile gradient at a constant 1 mL·min^{-1}. A gradient of water (A) and acetonitrile (B) was employed to separate the compounds by increasing the proportion of solvent B from 2% to 20% over 25 min and then switched to an additional linear gradient of 20% to 100% of solvent B up to 35 min. The glucosinolate analysis was replicated three times.

3. Results

3.1. Developing Stable Inbred

Multiple plants were produced from the cultured hybrid ovules (F_1) between jombaechoo and gaetmoo. Eight (F_1) of 13 plants from 07OV-124 to 07OV-136 were transplanted into soil after acclimation, grown in the greenhouse, and

treated with colchicine. Most plants produced large amounts of pollen. One plant, 07OV-125, produced only one seed (F_2) from self-pollination and the other seven plants produced no F_2 seed. However, 15 hybrid seeds from 1 to 4 of 9 mates designated F_1F_1 were produced by reciprocally cross-pollinating the plants with the cultivar BB#1. One plant, 07OV-127, subjected to microspore mutagenesis for stabilization, produced a few embryos and two plants (designated F_1Mn_1) were acclimated.

All seeds harvested from self-pollinating the F_1 plants (one seed, F_2) and cross-pollinating the F_1 plants with BB#1 (15 seeds, F_1F_1) were planted and grown together with the two microspore-derived plants (F_1Mn_1). All plants produced large amounts of pollen. However, one F_2 plant did not produce any seed from self-pollinations made on more than 100 flowers and cross-pollinations made on 45 flowers distributed over 10 branches over a period of 6 d. It was determined to be female sterile. Two plants (07OV-127Mn-1 and 07OV-127Mn-2) derived from microspore mutagenesis did not produce seed from self-pollinations despite a total of 1,099 pollinations. However, these plants produced some many seeds in reciprocal crosses with several accessions of ×*Brassicoraphanus*. These results indicated that both 07OV-127Mn-1 and 07OV-127Mn-2 were self sterile, and not stable. Only 5 of 15 F_1F_1 plants produced self seeds (F_1F_2). Seed yields were poor, at 1 - 3 seeds per plant, with the exception of (BB#1x07OV-128)-3, which produced 29 seeds. On the contrary, the yield of hybrid seeds ($F_1F_1BC_1$) of three plants crossed with BB#1 was generally as high as 6 - 17 seeds per combination (Table 1). The reason that fewer seeds were produced from self-pollination than cross-pollination is unknown.

Table 1. The number of seeds and plants obtained from pollination and microspore mutagenesis in 2nd generation and the number of seeds in 3rd generation of hybrid plants (F_1) between land races of *B. rapa* (jombaechoo) and *R. sativus* (gaetmoo).

Original Plant code	2nd generation		3rd generation		
	Pollination or mutagenesis	Number of seeds or plants	Obtained seeds		
			Singular code	Self	Cross with other x*Brassicaraphanus*
07OV-125	self	1	1	0	0
07OV-127	Mn	2	1	0	212
			2	0	24
07OV-128	Cross to BB#1	5	1	1	-
			2	1	12
			3	2	17
			4	29	Used to Mn
07OV-136	Cross to BB#1	1	1	3	14
Total		2 plants + 7 seeds	8 plants	5 lines 36 grains	5 lines 279grains

Mn: Microspore culture with mutagen NMU of 0.01 μM. Pollination was done to more than 15 flowers for each mate.

An F_1F_1 plant, (BB#1x07OV-128)-4, produced the greatest amount of seed from self-pollinations and was subjected to microspore mutagenesis. Twenty seven and 38 plants from plots treated with and without mutagen, respectively, were grown until flowering. Only 7 and 8 plants, among the 21 and 28 pollen-producing plants, respectively, produced seeds from self-pollination. However, only one plant derived from mutagen treatment and two plants derived without mutagen treatment in each plot were selected as being highly fertile, as they produced more than 10 seeds per plant and 1 seed per pollination on the highest branch (Table 2).

In the succeeding generation, two of the three high-seed-yielding plants were grown separately in a different net case of 1-m length × 1-m width × 2-m height and pollinated via bees for 20 d. From this trial, only one line originating from the NMU treatment produced an average of 35g (about 7.000 grains) of seeds per plant (Table 3). This seed yield was similar to that for BB#1. When grown in the field during the fall and spring seasons, the one line was highly uniform and different from BB#1 in morphology. Leaves were dark green and glossy, and longer and fewer in number than BB#1 leaves. Bolting time in spring was also much later than for BB#1 (Figure 1). This line was labeled BB#5 and designated for a cytology study, glucosinolate analysis and variety protection. Plant performance was stable for more than six subsequent generations. Microspore muta-

Table 2. The number of plants pollen-producing, fertile, sterile and high fertile in self pollination among microspore-derived plants with or without treatment of mutagen, NMU, to a hybrid (F_1 F_1) between 2 ×*Brassicoraphanus* accessions.

Line code	No. of plants			
	total	with pollen	fertile	high fertile
(BB#1x07OV-128)-3MC	38	28	8	2 (7^{th} and 35^{th} plants)
(BB#1x07OV-128)-3Mn	27	21	7	1 (2^{nd} plant)※
Total	65	49	15	3

MC: Microspore culture. Mn: Microspore culture with mutagen NMU of 0.01 μM. The line, BB#1 is a stable cultivar and the plant 07OV-128 is a newly synthesized unstable allopolyploid in ×Brassicoraphanus. ※: Stabilized.

Table 3. Fertility of 3 plants selected for high seed yield among microspore-derived plants of an F_1F_1 hybrid in ×*Brassicoraphanus*.

Plant code	Fertility at $F_1F_1Mc_1$ generation			Fertility at $F_1F_1Mc_2$ generation
	No. of pollinated flowers	No. of obtained seeds		
		Average	Highest	
(BB#1x07OV-128)-3MC-7	608	97	1.2	0.3 gr/plant
(BB#1x07OV-128)-3MC-35	164	86	3.0	3.5 gr/plant
(BB#1x07OV-128)-3Mn-2	366	361	2.5	35.0 gr/plant

MC: Microspore culture. Mn: Microspore culture with mutagen, NMU of 0.01 μM. Fertility in next generation: pollinated with bees in the net case of 1 m length ×1 m width ×2 m high for 20 d.

Figure 1. Morphology of BB#5 compared to BB#1. Right is BB#1 and left is BB#5 in each figure. Left: uniformity on the field in fall season. Right Length of flower stalk grown in spring season.

genesis with NMU was confirmed as a practical method of stabilizing allopolyploids between *B. rapa* and *R. sativus*. BB#5 was registered after 2 year's examination in 2015.

3.2. Cytology

The unstable line BB#4 and stable cultivar BB#1 were compared cytologically with BB#5. Various irregular divisions in meiosis of chromosomes were observed in all lines. However, the numbers of cells undergoing abnormal division were different between stable and unstable lines. The stable BB#1 and BB#5 lines were 10% and 12%, respectively, in terms of irregularities from prophase to anaphase, whereas the unstable line BB#4 was 57.4% irregular from prophase to anaphase (**Table 4**). GISH analysis identified 19 chromosome pairs, 10 from the AA genome of *B. rapa* and 9 from the RR genome of *R. sativus* (**Figure 2**). These results indicated that the BB#5 of baemoochae undergoes diploid-like division during meiosis [2].

3.3. Glucosinolate Analysis

The total glucosinolate content of BB# 5 was slightly lower than that of BB#1 (4.64 and 4.94 mg·g^{-1} dry weight [D.W.], respectively). The content of glucoraphenin, a precursor of sulforaphene, of BB#5 was similar to that of BB#1, at 1.57 and 1.52 mg·g^{-1} D.W., respectively (**Table 5**).

4. Discussion

Inter-generic allopolyploids have not evolved into stable crops despite numerous attempts [2]. Chen and Wu (2008) [7] reported stable progeny of ×*Brassicoraphanus*, an allopolyploid between *R. sativus* and *B. oleracea*, which were derived from mass selection for 10 generations, although they were not developed into cultivated crops. Lee *et al.* (2011) [8] developed stable progeny of ×*Brassicoraphanus*, an inter-generic allopolyploid between *B. rapa* and *R. sativus*, through induced mutation using microspore culture. The result, "baemoochae", became a new vegetable crop [12] and was released as cultivar BB#1 [10]. Another stable

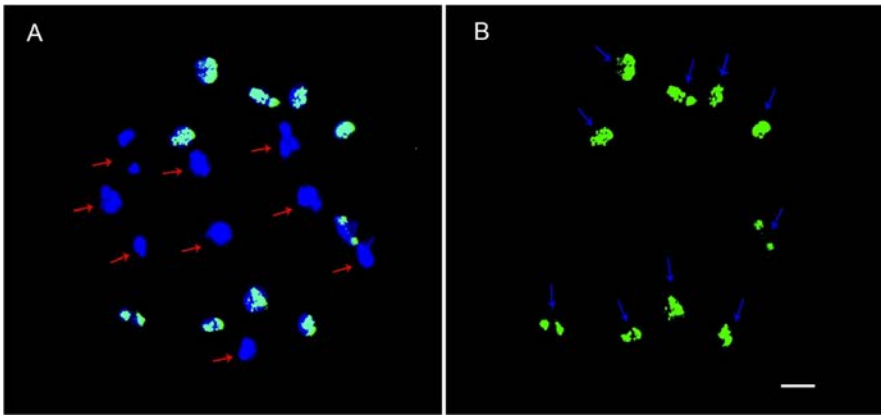

Figure 2. Genomic *in situ* hybridization on the meiotic chromosome pairs of BB#5 line in ×*Brassicoraphanus.* Meiotic chromosome pairs of *Raphanus sativus* (RR genome) are indicated with red arrows which show 9 bivalent chromosome pairs as expected to be derived from the RR genome (A) and those of *Brassica rapa* (AA genome) are shown as green signals indicated with blue arrows which show 10 bivalent chromosome pairs as expected to be derived from the AA genome. Scale bar, 5 μm.

Table 4. The number of irregular division cells out of 500 cells in 3 inbreds of ×*Brassicoraphanus* at different stage in meiosis.

Lines	Prophase(diakinesis)			Metaphase		Anaphase		Total in 500 cells (%)
				I	II	I	II	
	sticky	rod and ring	laggard	laggard		bridge and laggard		
BB#4	35	18	22	154	8	49	1	287 (57.4%)
BB#1	4	8	12	15	6	5	0	50 (10.0%)
BB#5	6	0	5	17	5	27	0	60 (12.0%)

BB#4 = unstable line. BB#1 and BB#5 = stable lines.

Table 5. Content of total glucosinolate and glucoraphenin in seed of baemoochae BB#5 and BB#1 (mg/g fresh weight).

Glucosinolate	BB#1	BB#5	Remark
Total glucosinolate	4.94	4.64	The results are mean of 3 replications
GRE (glucoraphenin)	1.52	1.57	

line, BB#5, was also a commercial crop, confirming the effectiveness of microspore mutagenesis for stabilization of ×*Brassicoraphanus*. These results indicate that an evolution to speciation in allopolyploids of *Brassica* and *Raphanus* has been accomplished and that subsequent development of improved cultivars is feasible.

Diploid-like division in meiosis of allopolyploids was hypothesized to result from chromosomal rearrangement with genomic changes [20] [21]. The exchange of homoeologous chromosomes, and missing and duplicated homoeologous genes, without a differential change in the expression of homoeologous sets of genes, was required for this chromosomal rearrangement [22] [23] [24].

The morphological characteristics of BB#1 and BB#5 were similar to those of their respective wild accessions, making it difficult to distinguish them. It is presumed, therefore, that microspore-holding homoeologous chromosomes were rearranged in the stable accessions through microspore mutagenesis as haploids, followed by chromosome duplication without changes in whole expression of homoeologous sets of genes.

Based on these results, it seems that another ×*Brassicoraphanus* cultivar could be developed from the combinations *R. sativus* and *B. nigra*, one of the 3 basic genomes in *Brassica* crop, by either induced mutation or mass selection. A cubic triangle, with *R. sativus* on the apex of the U's plane triangle of *Brassica* species [25], would be accomplished with three synthetic ×*Brassicoraphanus* on the three sides (**Figure 3**). This accomplishment would attract the attention of scientists aiming to produce improved *Brassica* cultivars.

Allopolyploids in plants are known to degenerate gradually by irregular division of chromosomes during meiosis [7] [26] [27]. Such unusual division should disturb the diploid-like division, which is a fundamental requirement in stabilizing allopolyploids [2]. The rates of abnormal chromosomes in meiosis were confirmed to be 10% and 12% in the stable lines BB#1 and BB#5, respectively, and 57.5% in the unstable line BB#4. These rates might be criteria for determining the stability of allopolyploids, although the true threshold has not been established. The rate of laggard cells in metaphase was as high as 31.0% (154 of 500 cells) in the unstable line, compared to around 3% (15 cells in BB#1 and 17 cells in BB#5 of a total of 500 cells) in the stable lines. The rate of laggard cells in metaphase may also be used as another standard to determine the stability of an allopolyploid.

Sulforaphene, a hydrolytic product of glucoraphenin, is a major isothiocya-

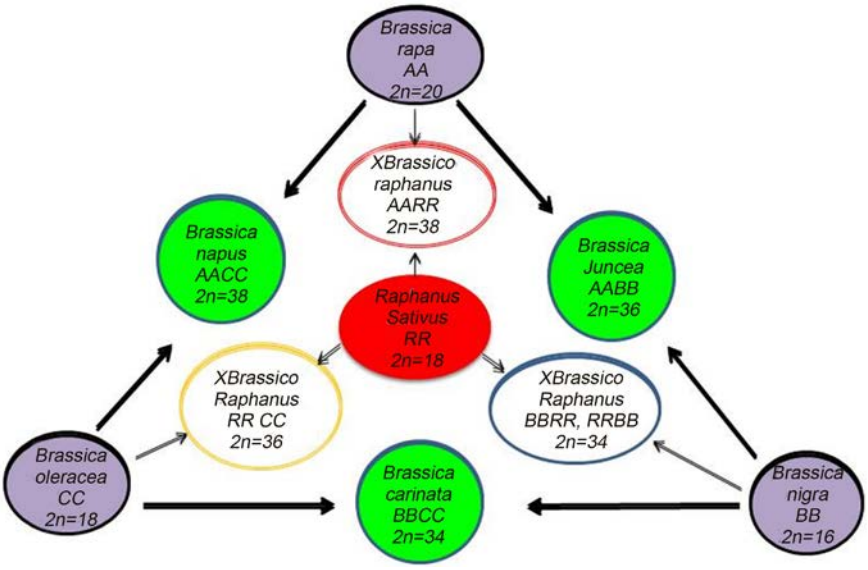

Figure 3. A cubic triangle with *R. sativus* on the apex of the U's plane triangle of *Brassica* species. The crosses between 3 basic genomes of the U's triangle and R. sativus would create 3 different species in ×*Brassicoraphanus.*

nate in baemoochae seeds [9]. Many studies have reported biological effects of sulforaphene, including antimicrobial, antifungal, antibacterial, nematocidal, and allelopatic properties [28]-[33]. The total glucosinolate content of BB#5 was slightly lower than that of BB#1, while the glucoraphenin content of the two lines was similar. This result indicated that baemoochae cultivars might perhaps be high levels in general of the anticancer and antibacterial substance glucoraphenin.

Two allopolyploid cultivars from the *B. rapa* and *R. sativus* cross and a stable allopolyploid line from the *R. sativus* and *B. oleracea* cross are currently available for field production and scientific studies. These cultivars differ in terms of their genomes and chromosome numbers. According to Williams' suggestions [34], which are the capital letter for the cytoplasmic genome and the small letter for the nuclear genome in nomenclature, cultivars from the *B. rapa* and *R. sativus* cross have an Aaarr genome and the cultivar from the *R. sativus* and *B. oleracea* cross has an Rccrr genome. Their chromosome number are 2n = 38 in the former and 2n = 36 in the latter. Therefore, these cultivars should be allotted distinct scientific names to avoid confusion with ×*Brassicoraphanus* Sageret [3]. We would like to nominate ×*Brassicoraphanus koranhort* Sageret and Lee for the nomenclature of the baemoochae crop, an allopolyploid between *B. rapa* and *R. sativus*.

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