Purple BB#10 development in ×Brassicoraphanus koranhort using turnip-intercalated radish chromosomes

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Research Article

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Abstract

The nidomi cultivar (introduction number **166**) of *Brassica rapa* ssp. *rapifera* (turnip) was crossed, after emasculation, with *Raphanus sativus* var. *rafinistrum* lr. (landrace) gaetmoo **(**radish no. **45**) to breed a fine-root variety. One seed was harvested. The turnip had already been intercalated with B-genome (**166***) and one seed was not crossed and delivered chromosome pieces of *Raphanus* (**166****). The intercalation and absence of hybridization were confirmed by marker and chromosome investigations at the C_2F_2 generation, when two plants of **CMSBB#1-11 × 166**-1-1** (C_1F_2) produced only one and two seeds in a cross with **BB#1**. When cultivated, **CMSBB#1-7 × 166**-1-2** (C_2F_2) produced seeds at 21.7% and 20.0% the amount of standard **BB#1**, and some purple plants were present in the 21.7% line. The purple plants presumably reflect intercalation with radish chromosomes at the pollination stage of the intergeneric hybrid. Three selected purple plants exhibited abundant pollen and were fertile, although they had previously been crossed with **CMS-BB#1**. Therefore, the pedigree method could be carried out for these plants (C_2F_2), and the seed production showed great improvement at the C_2F_4 generation. The purple plant was named **BB#10**; the pigment, glucoraphenin and glucoraphasatin, and chromosome constitution (C_2F_5) were analyzed. **Purple BB#10** was registered in 2019 (C_2F_6).

Introduction

The synthesis of intergeneric plant hybrids is important for the investigation of botanical evolution, broadening of germplasms, acquisition of new characters in agriculture, and development of new food materials (Banga et al. 2003; Bang et al. 2007; Jeong et al. 2009). Some researchers have reviewed interspecific and intergeneric hybrid breeding in the cruciferous group (Prakash et al. 2009; Kaneko and Bang 2014; Katche et al. 2019). A hybrid between *Raphanus sativus* and *Brassica oleracea* was first bred by Sageret (1826, cited from Prakash et al. 2009), then by Karpechenko (1924, 1928) and McNaughton (1973, 1979). Finally, Chen and Woo (2008) stabilized the intergeneric hybrid by using a deliberate pedigree method. A hybrid between *Brassica rapa* and *R. sativus* was first developed by Terasawa (1932), then by Takeshita et al. (1980), Dolstra (1982), Been and Park (1983), Cho (1986), Lange et al. (1989), Lou et al. (2017), and Jin et al. (2020). We obtained hybrid plants from *B. rapa* ssp. *pekinensis* and *R. sativus* that had been adapted using the ovule culture technique (Lee et al. 1989, 2002), which was developed by Been and Park (1983) and improved by Cho (1986). The hybrid was finally stabilized by microspore mutation (Lee et al. 2011, 2017).

The hybrid between ssp. *pekinensis* and *R. sativus* possesses very tender and soft tissue, good taste, high amounts of nutritional inorganic and organic substances (Lee et al. 2012; Zhang et al. 2019), and high anti-cancer and anti-bacterial isothiocyanates (Lim et al. 2009; Bhandari et al. 2015; Han, 2018; Nugroho et al. 2020). Therefore, **BB#12** was registered for variety protection under the cultivar name **BB#1** with the crop name baemoochae in 2009 (No. 2887) after stabilization. Subsequently, **BB#5** was developed (Belandres et al. 2015; Lee et al. 2017) and registered in 2015 (No. 5787). It maintains a darker green color, is taller, and has extremely late flower stock initiation in the spring season, compared with **BB#1**.

The scientific name proposed for the baemoochae at the suggestion of Toxopeus (1985) is ×*Brassicoraphanus koranhort* Sageret and Lee (Soo-Seong).

The baemoochae root was swollen, with a radish-like appearance, and had slim lateral roots before stabilization, although it was not uniform among individuals. However, stabilization led to a small and straight root (Lee et al. 2011). Because baemoochae have abundant anti-cancer and antibacterial glucosinolates in the root (Lim et al. 2009; Nugroho 2020), breeding of the fine root has been a source of concern. An important method involves attempts to first develop a hybrid between turnip and radish, a task at which Dolstra (1982) has already succeeded by obtaining mature seeds, and then hybridizing this hybrid with baemoochae **BB#1**. However, unexpectedly, purple plants were present in the C_2F_2 generation during the breeding procedure; such plants were fixed as a purple cultivar. Eventually, a **purple BB#10** maintaining the purple color in the top part along with the fine root was developed and registered as a variety of baemoochae in 2019 (No. 7469).

Materials And Methods

-Development of the “Purple BB#10” baemoochae

We intended to use *B. rapa* ssp. *rapifera* cv. nidomi (no. **166**; genome symbol: AA) and *R. sativus* var. *rafinistrum* lr. (landrace) gaetmoo (**45**, genome symbol: RR). The cultivars **nidomi** and **gaetmoo** were introduced from the National Institute of Horticulture and Herbal Science and the Germplasm Center, Office of Rural Development, in 2005 and 2003, respectively. Cultivar **nidomi** was emasculated before pollination with **gaetmoo**, but **gaetmoo** was not castrated to ensure clear recognition of the difference between the two self-incompatibility (SI) plants in the intergeneric cross. Because one plant (**166 × 45**) obtained from emasculated turnip and six plants (**45 × 166**) obtained from non-castrated gaetmoo showed different morphologies, the six plants from radish were discarded after successful multiplication of the one plant for the next generation.

Two plants were generally chosen for seed production of the next generation from the fall crop culture to mitigate the risk of a single plant dying during the progression. Two plants of **CMSBB#1-11 × 166-1-1-1** produced only one and two grains of seed, respectively, at the C_2F_2 generation via the **BB#1** cross, whereas **CMSBB#1-7 × 166-1-1-2** produced 152 and 182 grains. Thus, the marker and chromosome observations were requested from two collaborators, Seoul National University and Sahmyook University. Because the turnip cultivar already had the intercalated B genome, an asterisk was added to the varietal introduction number (i.e., **166***) to distinguish it from the non-intercalated cultivar (**166**). When the turnip and radish were hybridized with each other, they sometimes did not cross, and some pieces of the counterpart chromosomes were provided. In such cases, two asterisk marks for turnip (i.e., **166****) and one asterisk mark for radish (**45***) were used. When purple plants occurred at the C_2F_2 generation, the cytoplasmic male sterility (CMS) of the chosen plants disappeared.

Therefore, a pedigree method was applied to develop the purple cultivar. Because both strains were identical and did not differ in terms of various traits in 2014, they were combined into one lineage, named "**BB#10"**, for further investigation and commercial sale. When **BB#10** was confirmed to be almost fixed, its bolting property was tested through sowing with **BB#1** and **BB#5** on March 15 and April 10, 2015, which were optimum times for spring cultivation. It has been impossible to grow **BB#1** in spring because the development of its flower buds initiates before plant maturation, and **BB#5** did not bolt, even with February sowing. We also investigated the root shape in autumn of the same year. In 2017, the purple color components, glucoraphenin (GRE) and glucoraphasatin (GRH), genomic *in situ* hybridization of the meiosis of **BB#10** were investigated or performed at Seoul National, Chung-Ang, and Sahmyook Universities, respectively. Cultivar **BB#10** was multiplied in the field for the sale of seeds in 2018. The seed was renamed **"Purple BB#10"** and was shared with the government for registration testing for varietal protection**.**

Marker test and fluorescence in situ hybridization (FISH) for mitosis

For marker design, leaves of the sample were finely ground with beads using a TissueLyser II (Qiagen, USA); genomic DNA was extracted using cetyltrimethylammonium bromide (Dolye and Doyle, 1987). Genome sequences of *B. rapa* and *R. sativus* were compared, and three primer sets were developed to amplify species-specific genomic regions. Primers PxB-A-2 and PxB-A-3 were designed to target chromosomes A1 and A8 of *B. rapa*, respectively; primer PxB-R-2 was designed to target chromosome R5 of *R. sativus*. The polymerase chain reaction (PCR) amplicon sizes of primers PxB-A-2, PxB-A-3, and PxB-R-2 were 785, 949, and 700 bp, respectively. The PCR was performed in a 20-µl reaction volume containing 1000 ng of genomic DNA, 1 unit of *Taq* polymerase (Takara Bio, USA), 0.25 mM dNTP, and 0.2 mM primers, with a 1 × PCR reaction buffer (Takara Bio) under the following conditions: denaturation at 95°C for 5 min, 30 cycles of amplification (95°C for 30 s, 60°C for 30 s, and 72°C for 1 min), and extension at 72°C for 10 min. The PCR products were stained using EcoDye (Biofact, Korea) in a 1.2% agarose gel.

For chromosome observation, harvested root tips were treated with 2 mM 8-hydroxyquinoline for 5 h at 18°C, fixed with aceto-ethanol (1:3 v/v), and stored in 70% ethanol at 4°C until further use. The somatic chromosome spread preparation (Waminal et al. 2012) and FISH (Waminal et al. 2018) procedures were performed with some modifications. Pre-labeled oligonucleotide probes for MsatA (A genome-specific microsatellite), MsatBR (B/R genome-specific microsatellite), and CentRs (R genome-specific microsatellite) were designed using the CLC Main Workbench (Qiagen) and synthesized by Bio Basic Inc. (Toronto, Canada) (Campomayor et al. 2021). Slides were imaged using a fluorescence microscope (100× magnification; BX53, Olympus, Tokyo, Japan) and a built-in charge-coupled device camera (CoolSNAP™, Boston Microscopes, MA, USA). Images were captured and analyzed with Genus software (version 3.1) using fluorochrome-specific filters and finalized in Adobe Photoshop CS6.

Leaf color component analysis

To determine the major anthocyanin components in the purple baemoochae, color pigments were extracted from two leaves of young and old × ***Brassicoraphanus koranhort*** cv. **BB#10** with a control of ***B. rapa*** cv. **Chifu-401,** ***R. sativus*** cv. **WK10039,** and ×***Brassicoraphanus koranhort*** cv. **BB#1**. Anthocyanin pigments were extracted using a solvent mixture solution of acetone: methanol: water: formic acid (40: 40: 20: 0.1, v/v/v/v) (Zifkin et al. 2012). To remove sugar from extracts, they were filtered through a Sep-Pak C18 cartridge (Waters Scientific, Ontario, Canada). Completely evaporated samples were dissolved in 2 N HCl for the hydrolysis of anthocyanins to anthocyanidins. The samples were incubated at 100°C for 1 h and quick-chilled in ice, then centrifuged and transferred to glass vials. The flow rate of the high-performance liquid chromatography (HPLC) system (Ultimate 3000, Thermo Dionex, USA) across a 5 µm × 250 mm × 4.6 mm VDS C-18 column was set to 0.8 mL min^{-1} (VDSoptilab, Germany) at ambient temperature. The injection volume was 10 µL. Anthocyanidins were detected at the wavelength of 520 nm using delphinidin chloride, luteolinidin chloride, cyanidin chloride, pelargonidin chloride, peonidin chloride, and malvidin chloride (Sigma-Aldrich, St. Louis, MO, USA) as standards.

-Analysis of GRE and GRH of BB#10

Because the baemoochae seed glucoraphenin (GRE) and glucoraphasatin (GRH) were > 90% (90.2%), the levels of these substances were analyzed. Intact GRE and GRH were extracted as previously described (Ito and Horie 2008), with modifications. Lyophilized samples were ground in 70% methanol, and the suspension was incubated at 75.0°C for 20 min to inactivate the hydrolytic enzyme. The samples were centrifuged at 800 × *g* for 10 min, and the supernatants were evaporated to dryness and dissolved in methanol. The methanol extracts were filtered using a 0.45-µm polyvinylidene fluoride syringe filter (Acrodisc LC 13-mm syringe filter, Pall, NY, USA). The filtrates were analyzed using a Gilson HPLC system with, a C18 reverse phase column (Zorbax Eclipse XDB-C18, 4.6 × 250 mm i.d., 5-µm particle size; Macherey-Nagel, Düren, Germany) and the ultraviolet detector set at 229 nm. The mobile phase solvent system was 65% acetonitrile containing 2.5 mM tetrakis (decyl)ammonium bromide as an ion-pairing reagent. To identify the peaks corresponding to GRE and GRH, HPLC-(ESI) mass spectrometry (MS) analysis was performed using an Accela HPLC system coupled with a mass detector (ATQ Velos; Thermo Scientific, Waltham, MA, USA) coupled with a mass detector (ATQ Velos) and a Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA). The capillary and source heater temperatures were 275°C and 250°C, respectively. The flow rate was 0.2 mL min^{-1}, and the mass was scanned from 150 to 2,000 m/z.

BB#10 meiotic chromosome observation

Meiotic chromosomes were prepared as described by Belandres et al. (2015), and slide pretreatment was performed as described by Alexandrov et al. (2016). Genomic DNA (gDNA) was extracted from young leaves using cetyltrimethylammonium bromide (Allen et al. 2006; Zhou et al. 2019). Then, genomic probes and blocking DNA were developed as previously described (Melo et al. 2015; Razumova et al. 2016), with modifications. Briefly, the genomic DNA of *B. rapa* was labeled with DIG-11-dUTP (Roche, Germany) by nick translation, whereas the unlabeled sheared gDNA of *R. sativus* was used as blocking

DNA. The GISH mixture contained 50% formamide, 10% dextran sulfate, 2× SSC, 50 ng μL^{-1} probe DNA, and 5 ng μL^{-1} blocking DNA. The mixture was pre-denatured at 90°C and incubated on ice for 10 min. Approximately 40 µL was pipetted onto the slides, denatured at 80°C for 2.5 min, and hybridized overnight at 37°C. On the following day, stringency washing and dehydration in an ethanol series were performed (Pellerin et al. 2019; Peniton et al. 2020). DIG-labeled probes were detected using anti-DIG-FITC conjugate (Sigma, USA) and counterstained with a 40-µL aliquot of 1:100 DAPI (f.c. 1 $\mu g \cdot mL^{-1}$ [Roche, Germany]) in Vectashield (Vector Lab., Inc., USA). Finally, chromosomes were examined under a fluorescence microscope (Olympus BX51) equipped with a charge-coupled device camera (CoolSNAP cf). Probes were MsatA to discriminate A-genome chromosome subtelomeric FISH signals, MsatBR to discriminate B- and R-genomes with different signal distribution intercalary FISH signals, and CentR to discriminate R-genome chromosome centromeric FISH signals.

Results

-Development of “Purple BB#10” baemoochae

The procedures from obtaining intergeneric F_1 (**166 × 45** and **45 × 166**) to the 4^{th} generation, the generation in which purple plants occurred for the first time, are shown in Table 1. One mature seed was harvested from the hybridization of 13 emasculated buds of cv. nidomi and lr. gaetmoo; six seeds (**45 × 166**) were obtained in the reciprocal cross without castration (F_1). The strong SI plants of nidomi plants, which were segregated into strong and weak plants, were crossed with radish, which was also a strong SI plant (Appendix Table 2). When the two hybrids (**166 × 45** and **45 × 166**) were cultivated, one **166 × 45** plant, (**166 × 45)-1,** bloomed with yellow flowers and produced abundant pollen; four of six **45 × 166** plants bloomed with radish-like flowers and surprisingly, also produced abundant pollen. The (**166 × 45)-1** plant produced 153 seeds, (**166 × 45)-1-1**, including 81 and 72 seeds from 72 cases of bud self-pollination (BS) and 8 cases of flower self-pollination (FS), respectively (F_2). Although the seed yield increased from one to 153, it remained low, with 1.1 grains of seed per pollination at BS and 4.0 at FS. Thus, FS was much better than BS, which contrasts with the case of the previous generation. The property of self-compatibility produced the effect of a real hybrid between turnip and radish, although the hybrid had yellow flowers and abundant pollen production, despite intergeneric hybridization. Moreover, the plant was obtained from emasculated turnip. The four radishes × turnip plants were discarded; therefore, the plants continued to exhibit strong SI, similar to the female radish with non-castrated anthers at cross-pollination (Appendix Table 3).

Two F_2 plants, (**166 × 45)-1-1** and (**166 × 45)-1-2**, were chosen randomly from the multiplied line of 40 cultivated plants for the fall crop. They were self-pollinated to produce F_3 seeds (FS) and crossed with two **CMS-BB#1** plants, **CMS-BB#1-11** and **CMS-BB#1-7,** to simultaneously induce respective **CMS hybrids** (F_2C_1). The seed yield was much improved in FS with 1.087 and 875 grains of seed and 8.8 and 5.6 grains per pollination, respectively. Abundant seed was also produced in the cross with **CMS baemoochae**: 700 and 1.032 grains of seed with 10.2 and 8.6 grains per pollination, respectively. Those

two lines were crossed with the **11th** and **7th CMS plants**, **CMS-BB#1-11 × (166 × 45)-1-1** and **CMS-BB#1-7 × (166 × 45)-1-2**, on different branches; they were grown for the fall crop with 24 plants each. The **CMS-BB#1-11 × (166 × 45)-1-1** line was uniform and had medium vigor, many lateral shoots, a broad midrib, and a large root. The **CMS-BB#1-7 × (166 × 45)-1-2** line was poorly pure, had strong vigor, no lateral shoots, a round midrib, and a medium-size root. Surprisingly, distinct appearances were observed in the two combinations (Table 4), although they had been crossed with the same **CMS plant.** The cause of these different appearances could not be determined.

Two plants from each of the similar baemoochae were selected for two combinations (i.e., a total of four plants; F_2C_1), for use as seed production plants. When flowers bloomed in the next spring, the four plants produced no pollen, as expected. However, the two plants hybridized to the **11th CMS plant**, **CMS-BB#1-11 × (166 × 45)-1-1**, bloomed with uniformly yellow flowers; the other two plants crossed with the **7th CMS plant**, **CMS-BB#1-7 × (166 × 45)-1-2**, had impure colored white and beige flowers. Without any interest in flower color, it was desirable to breed another **CMS-BB#1** that passed through the turnip and gaetmoo hybrid. Two sets with three plants, consisting of the **11th** and **7th CMS plants** and **BB#1**, were placed together into two different small net cages to produce seeds under the natural condition with bees, respectively. When the harvested seeds were confirmed, the plants crossed with the **11th** and **7th CMS** and **BB#1** had produced 1, 152, and 700 grains, respectively, in the first net cage; they had produced 2, 182, and 910 grains, respectively, in the second net cage (F_2C_2). Plant **(166 × 45)-1-1** produced 700 grains with 8.6 seeds per pollination in the cross with **CMS-BB#1** in the previous generation. However, two selected plants of the progeny, {(**CMS-BB#1-11 × (166 × 45)-1-1**})**-7 and {(CMS-BB#1-11 × (166 × 45)-1-1}-6**, produced only 1 and 2 seeds, respectively. Nidomi turnip (**166-1-1-1**), gaetmoo radish **(45-4-4-1-1**), and the reciprocal crosses of the hybrid were therefore sent to Seoul National University for marker investigation and Sahmyook University for chromosome investigation.

Marker test and chromosome configuration at mitosis

In the marker test (Figure 1), radish and turnip were regarded as themselves; the combinations of turnip × radish and radish × turnip were not crossed between the two parents. About 10 turnip chromosomes already showed intercalation with B-genome pieces in the chromosome observation (i.e., **166*)** (Figure 2). Radish chromosomes were intact before pollination, **45**, but they were sandwiched with pieces of turnip chromosome after hybridization (Appendix Figure 3), **45***, although these had already been discarded. R-genome-intercalated turnip chromosomes are almost impossible to distinguish from the original turnip chromosomes because they have the same orange color. Presumably, turnip chromosomes would be sandwiched with radish chromosome pieces, additionally including the purple color, **166****, when the combination of turnip × radish was formed (Figure 4). If turnip chromosomes had not intercalated with B-genome pieces and crossed with gaetmoo radish, the turnip chromosome pieces would exhibit an orange color without B-genome and the hybrid would show a purple color.

Although the two lines crossed with the **7th CMS hybrid** produced only 152 and 182 seeds, 21.7% and 20.0% of the values of the normal **BB#1** (700 and 910 seeds), respectively, they were grown to observe

the appearance in the advanced generation. Twenty-two plants each were grown as seeding numbers **12BR-166**, for the combination of {**CMS-BB#1-7 × (166** × 45)-1-2}-3 × BB#1-2-2,** and **12BR-167** for the combination of {**CMS-BB#1-7 × (166** × 45)-1-2}-4 × BB#1-2-5** in the autumn of 2012. Surprisingly, in seeding number **12BR-166**, 16 of 22 plants had purple color. In seeding number **12BR-167**, all plants were green. The purple plants, segregating the purple vein, came from radish because **BB#1** was registered as the green cultivar; the root turnip had not shown purple color for more than five generations since its introduction in 2005. The combination of gaetmoo × turnip (gaetmoo had not crossed with turnip) was cultivated to reconfirm the result, and purple color arose from the purple vein (Appendix Figure 5).

Although it was a favorable color, the purple color was not uniform and its stabilization could be a concern in the future. From among the plants of similar baemoochae with dark purple color, **12BR-166-1**, **12BR-166-2**, **12BR-166-3**, **12BR-166-4**, and **12BR-166-5** were selected for initial seed production. In the next spring, these plants bloomed with completely white flowers, which had only been previously observed in × *Brassicoraphanus*. However, all were male and fertile, such that they produced abundant pollen. The CMS property had disappeared, as previously noted in the **BB#1** baemoochae (Appendix Table 5 and data not reported), and the pedigree method could be applied for fixation. Plants **12BR-166-1** and **12BR-166-3** died. Therefore, plant **12BR-166-4** was artificially prompted to undergo BS; the other two plants, **12BR-166-2** and **12BR-166-5**, were placed together in a small net cage and crossed with each other using bees. The seed production rates were much lower: 57 grains in the BS and 778 seeds in the cross pollination (FC) of the two plants (Table 6, F_3C_2). The low seed productivity was a second occurrence (i.e., the previous generation and this generation). When the BS of line **12BR-166-4** and the FC of line **12BR-166-2 × 12BR-166-5** were cultivated in the autumn, all plants had purple color and there were no differences between the two strains, although the color was uneven (Appendix Figure 6). Each single dark-purple plant and the similar baemoochae were selected again and placed into small net cages to produce seeds of the next generation. In total, 7,700 grains from the line **12BR-166-4-1** and 4,760 seeds from the line **(12BR-166-2 × 12BR-166-5)-3** were harvested (Table 7, F_4C_2). The seed yield was remarkably improved and was almost equivalent to the yield of **BB#1**; the pedigree method appeared to be successful. Presumably, the purple baemoochae had been stabilized in this generation.

Because the two strains were identical and did not differ in their various characters, they were combined into one lineage, named "**BB#10"**, for further investigation and commercial sale. **BB#10** had an almost uniform purple color and root shape; the seed yield was maintained during observations in 2015 and 2016 (Figures 7, 8, and 9). In particular, flower bud initiation was sufficiently late for growth in the spring season, as shown in the test involving **BB#1** and **BB#5** with March 15 and April 11 sowings, respectively. The flower branch of **BB#10** was short at harvest time on June 10, 83 days after seeding on March 15; no symptoms were observed on June 20, 70 days after seeding on April 11 (Figure 10). Cultivar **BB#1** had already flowered, and **BB#5** showed no symptoms of bolting. **BB#10** could possibly be grown with **BB#5** in spring and with **BB#**1 in autumn, ensuring year-round production of purple baemoochae. In 2017, the purple color components, GRE and GRH, and the genomic in situ hybridization for meiosis of **BB#10** were investigated at Seoul National, Chung-Ang, and Sahmyook Universities, respectively. When cultivar **BB#10**

was multiplied in the field for the sale of seeds in 2018, the number of harvested seeds was approximately 1,245 kg per ha, which was approximately the same as the amount for the **BB#1** baemoochae. The seed was shared with the government for a registration test for varietal protection after it had been renamed **"Purple BB#10"**; it was registered under the number 7469 in January 2019.

-Purple color component analysis

After sugar removal from the extracts and centrifugation, the purple pigments of young and old leaves of **BB#10** exhibited equal intensity (Figure 11). The other samples, **Chifu (CF)**, **Weongyo 39 (WK)**, and **BB#1 (BB1-G)**, showed white or yellow pigments. These results indicate that the purple color of cultivar **BB#10** could be attributed to anthocyanins. An assessment of six component anthocyanins revealed that cyanidin is the major type of anthocyanin in the samples studied (Table 8).

-HPLC analysis of GRE and GRH

Analyses of GRE and GRH, which are major glucosinolates in baemoochae seed, revealed that cultivar **BB#10** has similar or slightly higher concentrations in the outer and inner root and leaves, compared with **BB#1** (Table 9).

-Genomic *in situ* hybridization (GISH) for meiosis

BB#10 has n = 19 (2n = 38) chromosomes, similar to **BB#1** and **BB#5** (Figure 12). The GISH analysis configurations of kimchi cabbage and radish are n = 10 (2n = 20) and n = 9 (2n = 18), respectively.

Discussion

Marker and chromosome investigations of the intact materials revealed that radish, **45**, had undamaged chromosomes but that turnip had intercalated B-genome chromosomes, **166***. The hybrids had inserted chromosomes of the mated males (**45***, **166****), although chromosome pieces of radish did not show in turnip (this would be indicated by green color at the centromere). The nidomi turnip cultivar with R-genome chromosome pieces exhibited purple color. Therefore, the purple color of the radish chromosome was located at an area slightly remote from the central region of the chromosome. Because the centromeric radish chromosome exhibited green color, the turnip chromosome containing purple color should partially exhibit green color if near the centromere.

Turnip in hybridization with radish also exhibited insertion of radish chromosome pieces that would be segregated as leaf vein color. The multiplied plant of turnip, **166**-1-1**, did not hybridize with **CMS BB#1–11** but exhibited androgenesis with **CMS BB#1–11**; thus, the nuclear material was turnip from the male and the cytoplasm was **CMS BB#1** from the female (Tsuda et al. 2011; Schwander and Oldroyd, 2016; Rivas-Sendra et al. 2019), although the seed yield was very high (8.6 grains per pollination). Turnip **166**-1-1** bloomed with yellow flowers and was CMS discriminated to the self-fertilized male or female. In the next generation, the female of **CMS turnip**, (**CMSBB#1–11 × 166**-1-1-1**)**-7** or **6**, produced only one or two grains of seed in hybridization with the normal **BB#1** plant. The small or large chromosome

number influenced the seed production rate of the female: the small chromosome number turnip with 2n = 20 had fewer seeds than did the large chromosome number female, the normal **BB#1** plant with 2n = 38 (Lee et al. 2012). The **166**-1-1-2** plant, representing another line of turnip, was crossed with **CMS BB#1** and produced seeds at rates of only 21.7% and 20.0%, respectively, compared with normal **BB#1** (700 and 910 seeds). Plants of the hybrid **CMS BB#1 × 166**-1-1-2** bloomed with white and beige flowers and produced seeds in the cross with normal **BB#1** with the large chromosome number, although they were produced in small amounts.

Turnip cultivated for root production (*Brassica*) was intentionally crossed with radish (*Raphanus*) (Dolstra, 1982; Lou et al. 2017; Jin et al. 2020) to develop a fine root cultivar in baemoochae. However, the turnip was not hybridized and remained as turnip with some intercalated radish chromosomes including the purple color; it was then crossed two times with baemoochae. The purple color was fixed and seed productivity was normal in 2015, and the baemoochae exhibited a fine root, although the root was slightly smaller than intended. In the future, root baemoochae could be developed by hybridization between a root vegetable such as turnip and baemoochae (Lee et al. 2012).

The three baemoochae varieties developed have been registered, and they have different typical characteristics. **BB#1** has a light-green color and weak heading ability. **BB#5** is dark green, has very late flower stalk development, and exhibits no heading ability. **Purple BB#10** has a purple color at the top and medium characters of flower stalk initiation. Therefore, these are useful germplasms for cultivar improvement.

Declarations

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Competing Interests

We hereby declare that authors have no pecuniary or other personal interest, direct or indirect,

any matter that raises or may raise a conflict.

Author e-mail address and contribution statement

S-S L: Writing manuscript

CY S:Work for ovule culture

TY K: General (plant growth) affair

J K: Interpretation data

H S:Marker design

J E P: Marker test and interpretation

S H Y:Marker make and test

J H H:Guide and consult for Marker test as a professor.

J Y: Glucosinolate analysis

N H: Glucosinolate analysis

J Kim: Guide and consult for glucosinolate analysis as a professor

H H Kim:Guide and consult for chromosome observation

F M:Chromosome observation

N B C: Chromosome observation

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1. Flow chart of intergeneric hybrids between *Brassica* and *Raphanus* from one seed of harvest (F_1) to purple plants showing (F_2C_2) [Z].

Year	Combination and pedigree	Seed yield (grains)		Remarks
		Total	Per poll.	
2009	166-1 × 45-1 (F_1)	1	BC: 0.08	Multiplied
	45-1 × 166-2 (F_1)	6	BC: 0.35	Discarded
2010	(166-1 × 45-1)-1 (F_2)	153	BS: 1.1 (81 seeds)	
			FS: 4.0 (72 seeds)	
2011	(166-1 × 45-1)-1-1 (F_3)	1.087	FS: 8.8	
	(166-1 × 45-1)-1-2 (F_3)	875	FS: 5.6	
	CMSBB#1-11 × (166-1 × 45-1) -1-1 (F_2C_1)	700	FC: 8.6	CMS introduction
	CMSBB#1-7 × (166-1 × 45-1)-1-2 (F_2C_1)	1.032	FC: 10.2	CMS introduction
2012 1case	(CMSBB#1-11 × (166-1 × 45-1) -1-1)-7 × BB#1-2 (F_2C_2)	1		Non-cultivated
2012 2case	(CMSBB#1-7 × (166-1 × 45-1) -1-2)-3 × BB#1-2 (F_2C_2)	152		Purple plants
	BB#1-2 (FS)	700		
	(CMSBB#1-11 × (166-1 × 45-1) -1-1)-6 × BB#1-5 (F_2C_2)	2		Non-cultivated
	(CMSBB#1-7 × (166-1 × 45-1) -1-2)-4 × BB#1-5 (F_2C_2)	182		Green plants
	BB#1-5 (FS)	910		

poll.: pollination.

166: Introduction number of Brassica rapa ssp. rapifera cv. nidomi: 05-80-166 (Omitted 05-80).

45: Introduction number of Raphanus sativus lr. gaetmoo: 03-80-45 (Omitted 03-80).

BC: bud cross. BS: bud self-pollination. FS: flower self-pollination. FC: flower cross.

(F_2C_1): First cross of F_2 generation of 166-1x45-1 to CMS BB#1.

(F_2C_2): Second cross of F_2 generation of 166-1x45-1 of CMS BB#1 to BB#1.

Appendix Table 2 Results of self-pollination of the two parents

Number of lines	SI activity	Number in bud (self)			Number in flower (self)		
		Buds (A)	Seeds (B)	B/A	Flowers (A)	Seeds (B)	B/A
09BRS-166-1-1-1	Strong	116	508	4.4	25	6	0.2
09BRS-166-1-1-2	Weak	138	715	5.2	28	156	5.6
09BRS-45-4-4-1-1	Strong	147	109	0.74	38	1	0.03

[Z:] *The introduction year and crop number of the two introduced plants were omitted.*

Appendix Table 3 Seed yields and self-incompatibility of each of two selected plants of gaetmoo × nidomi, (45-1 × 166-1)-1 and (45-1 × 166-1)-2, with no castration

Number of lines	Number in bud (self)			Number in flower (self)		
	Buds (A)	Seeds (B)	B/A	Flowers (A)	Seeds (B)	B/A
(45-1 × 166-1)-1	155	406	*2.62*	52	14	0.3
(45-1 × 166-1)-2	80	82	*1.0*	39	19	0.7

a; The introduction year and crop number of the two introduced plants were omitted.

Self-incompatibility of the two hybrid plants was very strong (0.3 and 0.7 grains of seed) although they were hybridized (FS) with turnip.

Table 4. Characteristics of cross combinations of different individuals of CMS BB#1 × *rassicoraphanus koranhort* to turnip and inbred as a check [Z].

Combination or inbred	Purity	Vigor	Lateral shoots	Midrib	Root	Root taste
CMS-BB#1-11 × (166-1 × 45-1)-1-1	Pure	Medium	Many	Like kimchi cabbage	Large	Sweet, crispy
CMS-BB#1-7 × (166-1 × 45-1) -1-2	Impure	Strong	No	Like turnip	Medium	Sweet
166-1-1	Pure	Weak	No	Like turnip	Small, round	Sweet, crispy

[Z]: *Self-pollination. Characteristics of two lines differed from each other.*

Appendix Table 5 Results of backcross to induce CMS of ×*Brassicoraphanus koranhort* from *Brassica juncea* CMS

Generation	Numbers		
	Plants investigated	Male sterile plants	Male fertile plants
BC_1F_1	43	7	36 (83.7%)
BC_2F_1	46	38	8 (17.4%)
BC_3F_1	30	18	12 (40.0%)
BC_4F_1	40	22	18 (45.0%)
Total	159	85	74 (46.5%)

Table 6. Harvested seed numbers from the self- and bee cross-pollinated selected purple plants.

Seeding number	Line code	Pollination technique	Number of seeds (grains)
12BR-166-4-1	(CMS 169**-1-2-3 × BB#1)-4-1 (S_1)	Hand pollination	57
12BR-166-2 × 12BR-166-5	(CMS 169**-1-2-3 × BB#1)-2 x (CMS 169**-1-2-3 × BB#1)-5 (C_1)	Net cage + bees	504 + 274

Table 7. Harvested seed numbers from self-pollination by bees of selected purple plants.

Seeding number	Pollination technique	Number of seeds (grains)
12BR-166-4-1-1 (S_2)	Net house + bees	7,700
(12BR-166-2 × 12BR-166-5)- 3 (S_1C_1)	Net house + bees	4,760

Table 8. Type of anthocyanin and amount of cyanidin in purple BB#10.

Accession	Type of anthocyanin	Height (mAU)	Area (mAU/min)	Amount (mg/L)
Chifu (Brassica)	-	0.000	0.000	0.00
Weongyo 39 (Raphanus)	-	0.000	0.000	0.00
BB#1 (× Brassicoraphanus)	-	0.000	0.000	0.00
Purple BB#10 young	Cyanidin	96.17	23.12	22.58
Purple BB#10 old	Cyanidin	134.34	32.98	32.21

Table 9. Contents of glucoraphenin and glucoraphasatin in BB#1 and BB#10 baemoochae.

Cultivar	Root or leaf	Content (mg/g D. W.)	
		Glucoraphenin (GRE)	Glucoraphasatin (GRH)
BB#1	Outer root	0.3 ± 0.0	6.3 ± 0.4
	Inner root	0.2 ± 0.0	3.5 ± 0.2
	Leaf	1.4 ± 0.1	4.8 ± 0.6
BB#10	Outer root	1.1 ± 0.1	6.1 ± 0.1
	Inner root	0.2 ± 0.1	5.0 ± 0.5
	Leaf	3.2 ± 0.3	5.0 ± 0.4

Figures

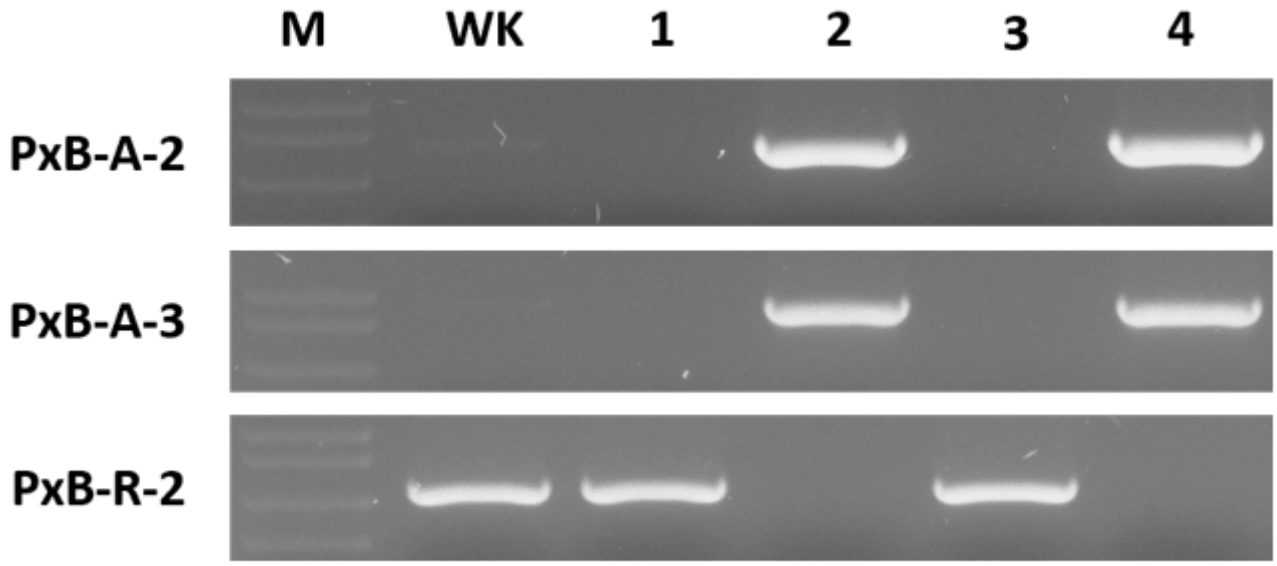

Figure 1

Results of marker tests on radish, turnip, and hybrids

a; PxB = Purple × green plants, A-2 and A-3 = Brassica rapa chromosome detection,

R-2 = Raphanus sativus chromosome detection, WK = Weonkyo (radish) DNA,

1 = radish DNA, 2 = turnip DNA, 3 = radish × turnip DNA, 4 = turnip × radish DNA

b; Radish DNA only was present as radish, and turnip DNA only was present as turnip.

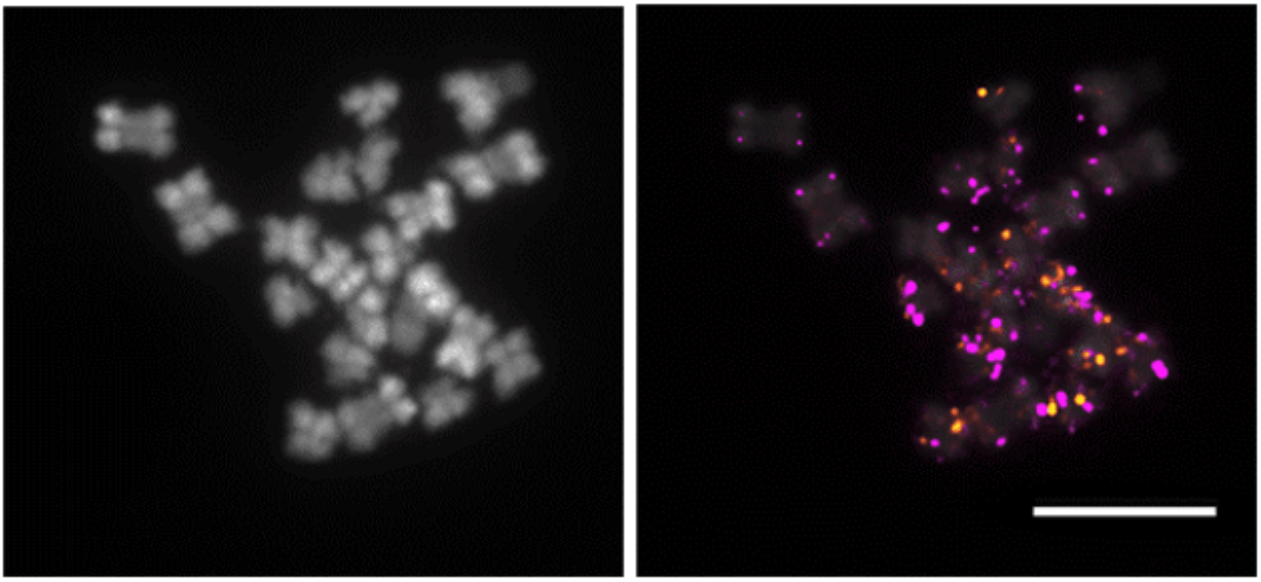

Figure 2

FISH metaphase spreads of *Brassica rapa* ssp. *rapifera* cv. nidomi (2n = 20)

a; *DAPI image (left). MsatA (pink) was detected in all 20 A-genome chromosomes. MsatBR (orange) was detected in 10 chromosomes with MsatA (right). Scale bar = 10 μm.*

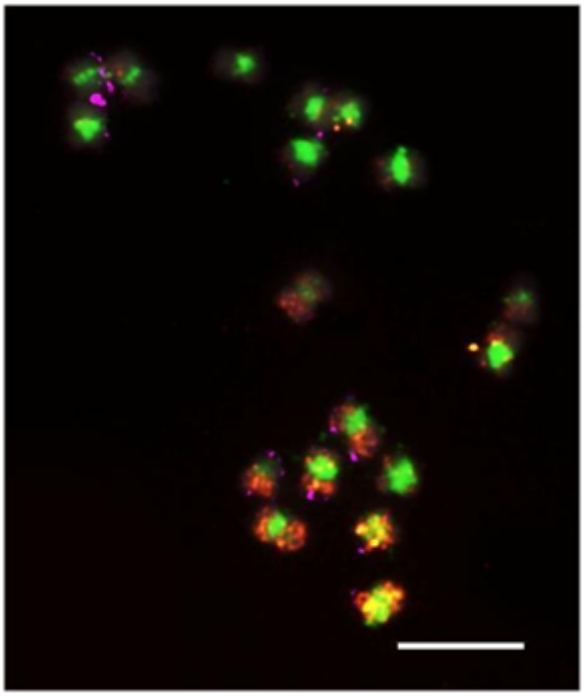

Figure 3

FISH metaphase spreads of radish. CentRs show the presence of A, B, and R genomes. Merged DAPI, MsatA (pink), MsatBR (orange), and CentRs (green) images. Scale bar = 10 μm.

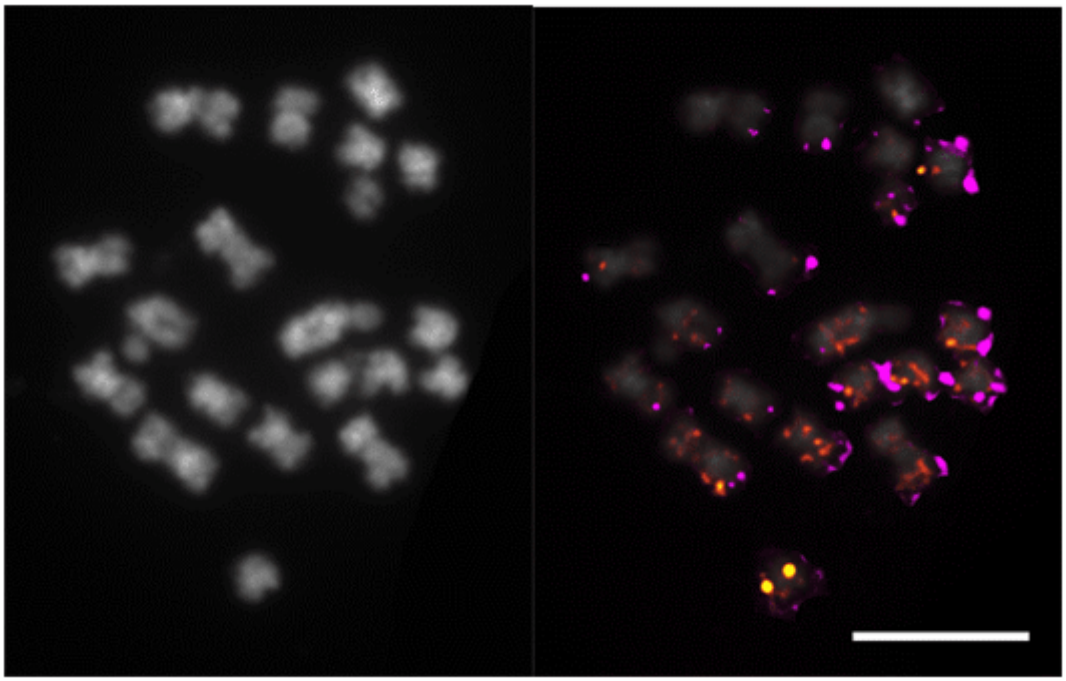

Figure 4

FISH metaphase spreads of *Brassica rapa* ssp. *rapifera* cv. nidomi (2n = 20), which might intercalate chromosomes of *Raphanus sativus* var. *rafinistrum* lr. gaetmoo (2n = 18) additionally

a; Raw DAPI image (left) and CentBRs (right, orange) showing the presence of A, B, and R-genomes.

MsatA (pink) and MsatBR (orange) images. Scale bar = 10 µm.

Figure 5

Purple color exhibited at the F_4 generation in the cross gaetmoo × nidomi (**45 × 166**). Gaetmoo had not crossed with turnip according to chromosome observation

Figure 6

Morphology of the purple plant at an early generation

Figure 7

Morphology and purple color level of Purple BB#10

a; Left: Autumn 2015; right: winter 2016, in PE house.

Figure 8

Root shape with direct seeding on the farm in the autumn of 2015

Figure 9

Seeds in pods of BB#10 grown in 2017

Figure 10

Bolting characters of BB#10 seeded on March 15 (left) and on April 11 (right)

a; BB#10 *(purple plant on the right in the left image) compared to* ***BB#5*** *(green plant on the left in the left image) showing bolting at harvest at approximately 83 days after seeding on March 15 but no bolting until 70 days (harvest day) after seeding on April 11.*

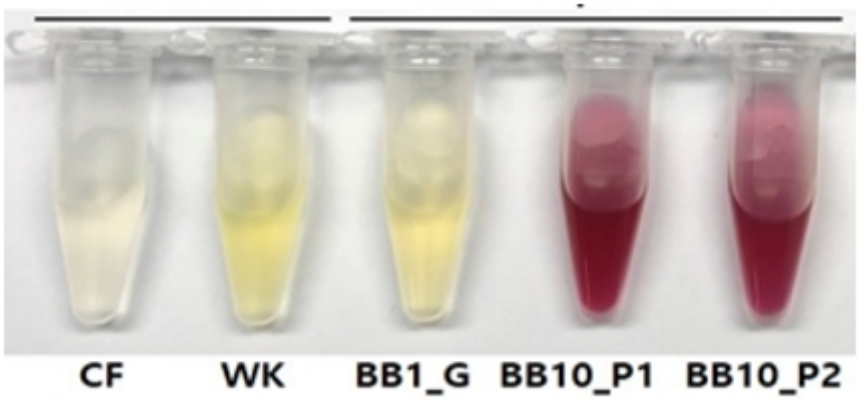

Figure 11

Samples transferred to glass vials after centrifugation and sugar removal

a; *CF: Chifu, WK: Weonkyo#39, BB1_G: BB#1, BB10_P1: young plant of BB10, BB10_P2: old plant of BB#10.*

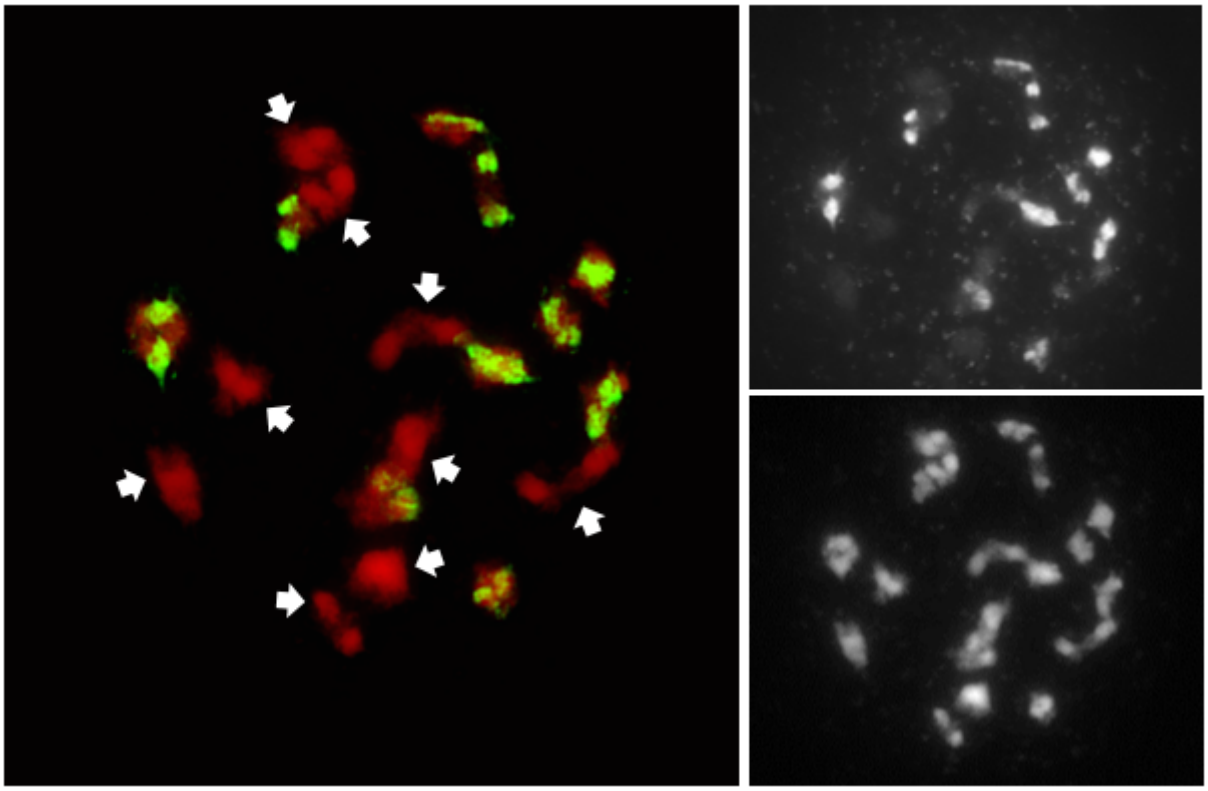

Figure 12

GISH analysis of ×*Brassicoraphanus* cultivar BB#10

(Left) merged image of BB#10, *Brassica rapa* (green) and *Raphanus sativus* (arrows) signals.

(Right, upper) Raw image of *B. rapa* signal. (Right, low) Raw image of DAPI staining indicating 19 bivalents.