Characteristics Analysis of F1 Hybrids between Genetically Modified *Brassica napus* and *B. rapa*

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Abstract

A number of studies have been conducted on hybridization between transgenic *Brassica napus* and *B. rapa* or backcross of F1 hybrid to their parents. However, trait changes must be analyzed to evaluate hybrid sustainability in nature. In the present study, *B. rapa* and transgenic (*BrAGL20*) *B. napus* were hybridized to verify the early flowering phenomenon of F1 hybrids, and F1 hybrid traits were analyzed to predict their impact on sustainability. Flowering of F1 hybrid has been induced slightly later than that of the transgenic *B. napus*, but flowering was available in the greenhouse without low temperature treatment to young plant, similar to the transgenic *B. napus*. It is because the *BrAGL20* gene has been transferred from transgenic *B. napus* to F1 hybrid. The size of F1 hybrid seeds was intermediate between those of *B. rapa* and transgenic *B. napus*, and ~40% of F1 pollen exhibited abnormal size and morphology. The form of the F1 stomata was also intermediate between that of *B. rapa* and transgenic *B. napus*, and the number of stomata was close to the parental mean. Among various fatty acids, the content of erucic acid exhibited the greatest change, owing to the polymorphism of parental *FATTY ACID ELONGASE 1* alleles. Furthermore, F2 hybrids could not be obtained. However, BC1 progeny were obtained by hand pollination of *B. rapa* with F1 hybrid pollen, with an outcrossing rate of 50%.

Introduction

The concern associated with the cultivation of genetically modified (GM) crops is the increase of weedy or invasive crops as the transgenes are transferred to related species [1,2]. The environmental risk of GM crops can be evaluated, based on the concept of substantial equivalence, by comparing GM and non-GM crops in a natural-like environment, and if any significant differences are observed, the likelihood of negative impact can be used as a measure of risk [3].
Also, the Cartagena Protocol on Biosafety III suggests that assessing the environmental risk of genetically modified organisms (GMOs) should be conducted by identifying the organisms’ new genotypic and phenotypic characteristics that could negatively impact the biodiversity of its potential habitat. In addition, although gene flow and introgression are natural processes, gene flow from transgenic plants to wild relatives complicates the potential introgression of new traits.

To date, researchers have attempted to predict the consequences of hybridization and introgression between transgenic crops and related species [4–8]. Halfhill et al. [7] reported that crop-weed hybrids have lower fitness and competitive ability than their parents, regardless of transgene introgression, and such decreases in hybrid fitness are suggested to result from the introduction of crop genes, rather than from the introduction of transgenes. Therefore, the fitness of hybrids derived from transgenic crops in natural ecosystems should be estimated with the sustainability of the transgenic hybrids in the ecosystem through the analysis of transgene introgression course and result, as well as trait changes in the hybrids, owing to the genetic load of crop-derived genes.

Considering the fact that Korea has imported transgenic *B. napus* seeds and has exported *B. rapa* seeds, it is very important to assess the gene flow of transgenic *B. napus* to *B. rapa* and to determine the ecological impacts of hybridization on the unintentional release of transgenes, in order to establish appropriate biosafety measures. *B. rapa* is one of the ancestral species of *B. napus*. A number of studies have shown that the two species can hybridize [9–12], that spontaneous F1 hybrids occur in nature [13], and that backcrossing occurs when the hybrids are grown in the vicinity of *B. rapa* [13–19]. Although the average fitness of the F1 hybrid and first backcross (BC1) progeny is generally low [9], *B. rapa*-like plants that contain transgenes can be found even after single backcrosses [10,18–23]. In addition, there have been more studies of hybrids between *B. rapa* and transgenic *B. napus* than hybrids between other *Brassicaceae* and transgenic *B. napus*; however, due to the genetic diversity of *B. rapa* the sustainability and ecological effects of crop genes introduced via pollen movement are poorly understood.

The regulation of flowering time is an important trait in terms of crop productivity. *AGAMOUS-LIKE 20* (AGL20) is known to encode a MADS-box transcription factor that integrates signals from multiple flowering pathways, including those related to photoperiod, temperature, hormones, and age [24]. A study in *Arabidopsis* demonstrated that AGL20 expression plays an important role in determining flowering time and affects many floral induction pathways [25]. When AGL20 from *B. rapa* was introduced into *B. napus*, the flowering time was advanced by up to 105 d, compared to non-transgenic *B. napus* [26], and although the early flowering transgenic *B. napus* has not yet been commercialized, it may be useful for assessing environmental sustainability, with regard to transgene expression and gene flow between transgenic *B. napus* and *B. rapa*.

This study aimed to investigate the expression pattern of target gene in the F1 hybrid between early flowering transgenic *B. napus* and *B. rapa*, analyze the environmental sustainability through analyzing morphological characteristics and fertility of F1 hybrid, and anticipate the unintended effects to the environment by the analyses of fatty acids of F1 seed. To do this, expression of target gene and other flowering-related genes was analyzed in F1 hybrids and parents. We also have compared the morphological characteristics of seeds, pollens and stomatal apparatus between F1 hybrids and parents. Fertility of F1 hybrids was investigated via self-pollination and backcrossing with *B. rapa*. Also, the content of erucic acid and nucleotide sequences of genes related to the synthesis of erucic acid were analyzed in the F1 hybrid seeds.
Materials and Methods

Plant materials

Early flowering transgenic *Brassica napus* L. ‘Youngsan’ (AACC, 2n = 38) was transformed with CAMV 35S-regulated bar and BrAGL20 [26], and *B. napus* L. ‘Youngsan’ and *B. rapa* L. ssp. pekinensis ‘Jangkang’ (AA, 2n = 20) seeds were obtained from the National Agrobiodiversity Center (Jeonju, Republic of Korea).

Outcrossing rate of F1 hybrid between *B. rapa* and transgenic *B. napus*

Cross experiments were conducted in the GMO greenhouse of NAAS (National Academy of Agricultural Science) located in Suwon, Korea. Interspecific crossability was determined using transgenic *B. napus* as the pollen donor and *B. rapa* as the seed parent, by means of artificial emasculation and crossing. For each cross combination, 100–200 flowers from 15 separate plants were crossed. Self-pollinated *B. napus* and transgenic *B. napus* were used as controls, and the pod number, number of seeds per pod, and seed weight were investigated after harvest. To obtain the F2 seeds 20 F1 plants were self-pollinated. In addition, BC1 progeny were produced using a F1 hybrid pollen donor and a *B. rapa* seed parent. Each of the 10 F1 hybrid and *B. rapa* were used to make BC1 seeds. For all of the progeny, crossability was calculated as the number of full seeds obtained per pollinated flower, and outcrossing rate was estimated by the survival (%) of seedlings after herbicide treatment. Briefly, seedlings were sprayed with 0.3% Basta (Bayer CropScience GmbH, Manheim am Rhein, Germany) at the 4–5 leaf stage and again 4 d later, and seedling survival was measured at 4–7 d after the second application.

Confirmation of hybridization by PCR

Genomic DNA was extracted from the leaves of plants, using the CTAB method [27], and PCR analysis was performed using primer sets for bar (forward primer: 5’-TCTGCACCA TCGTCAACCACCTACAT-3’; reverse primer 5’-CTGAAGTCCAGCTGCCAGAAAC CCA-3’) or the partial 35S promoter region and BrAGL20 (forward primer: 5’-GAGGCACAATCCCCTATC-3’; reverse primer: 5’-TCAGCTTTTCTTGAGAAACA AAGG-3’) . Because BrAGL20 gene has been cloned from *B. rapa* and transformed to *B. napus*, to detect the target gene of BrAGL20 from the F1 hybrid, a part of 35S promoter of the plant expression vector and BrAGL20 gene were used as the PCR amplification site. The PCR reactions contained 50 ng genomic DNA, 1× Taq buffer (Solgent, Republic of Korea), 50 μM dNTP mix, 1× Band Doctor (Solgent), 1.5 U Taq DNA polymerase (Solgent), and 25 pM of each primer in a total volume of 50 μl. The reaction conditions for bar amplification were as follows: pre-denaturation at 95°C for 4 min; 39 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 7 min. The reaction conditions for amplification of the partial 35S promoter and BrAGL20 were as follows: pre-denaturation at 95°C for 4 min; 39 cycles of denaturation at 95°C for 15 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. Amplifications were performed in a PTC-100 thermal cycler (Bio-Rad, Hercules, CA, USA), and the amplified products were electrophoresed on 0.8% agarose gels, stained with EtBr, and visualized using a UV transilluminator.

Confirmation of hybridization by flow cytometry

Fresh seedling leaves were chopped with a sharp razor blade in nuclei extraction buffer (solution A of the High Resolution Kit for Plant DNA; Partec Gmbh, Munster, Germany). After filtration through a 30-μm nylon sieve, the nuclei were stained with a solution that contained
4,6-diamidino-2-phenylindole-2HCl (i.e., DAPI; solution B of the High Resolution Kit for Plant DNA; Partec GmbH) and analyzed, using a PAS flow cytometer (Partec Gmbh). To estimate ploidy level, the fluorescent intensity peak of each sample was compared to the peaks of plants with known ploidy levels. One month later, leaves of the same plants were re-analyzed for confirmation.

Comparing of flowering time in transgenic *B. napus* and F1 hybrid
Seeds were sown in 4-inch pots containing a vermiculite. All plants were grown in a greenhouse under controlled temperatures (22/17°C day/night). The flowering responses were measured in terms of anthesis time (number of days until the first flower in the primary inflorescence opened). All experiments were done three times using at least 10 samples each time.

RNA extraction and real-time RT-PCR
Apical meristem samples were collected from *B. napus*, *B. rapa*, transgenic *B. napus*, and the F1 hybrid once a week for six weeks. Total RNA was prepared from the apical meristem samples using Trizol (Sigma-Aldrich, St. Louis, MO, USA), and 100 μg aliquots of the preparations were treated with RNase-Free DNase (Qiagen, Ontario, Canada). cDNA was generated by reverse transcribing 3 μg aliquots of the purified RNA in 50 μl reactions, using the ProSTAR First-Strand RT-PCR system (Stratagene, La Jolla, CA, USA) in the presence of oligo (dT) primers for 1 h at 37°C, and the reactions were terminated with heat inactivation at 70°C for 15 min.

To analyze the expression of the *BrAGL20* transgene and other floral-related genes (i.e., *AGL24*, *LFY*, and *AP1*), real-time RT-PCR was performed, using gene-specific primers:

- *(BrAGL20 forward: 5′-ATGGTGAGGGGCAAAACTCA-3′; BrAGL20 reverse: 5′- TCACCTTTCTTGAGAACA AG-3′; AGL24 forward: 5′-ATGGCGAGAGAAGATAAGG-3′; AGL24 reverse: 5′-TCATTTCCCAA GATGGAAGCCC-3′; LFY forward: 5′-ATGGA TCCTGAGGTTTCACG-3′; LFY reverse 5′-TTAA ACCCCAAAGCGTCCAGA-3′; AP1 forward: 5′-ATGGGAAGGGGTAGGGTTCAA-3′; AP1 reverse: 5′-TCATGCGGCCGAA GCAGCCAAG-3′).

All qPCR experiments were performed in a CFX real time system (Bio-Rad, USA) employing SYBR Green real-time RT-PCR Master Mix (Toyobo, Osaka, Japan). The reaction mixtures contained 5 μl diluted cDNA template (1:25), 10 μl 2× SYBR Green real-time PCR Master Mix (Toyobo), and 0.5 μM of each primer in a final volume of 20 μl. Amplification curves were generated using an initial denaturing step at 95°C for 3 min; followed by 40 cycles of 95°C for 15 s, 56°C for 15 s, and 72°C for 20 s; and melting curve analysis was performed at the end of the cycles (65 to 95°C at 0.2°C/s with continuous fluorescence readings), in order to ensure that single PCR products were obtained. To normalize the results, the *B. napus* actin gene (NCBI acc. no. KM881429) [28] was used as an internal standard. All real-time RT-PCR reactions were repeated in triplicate, using RNA isolated from three biological replicates, and the gene expression results were analyzed using CFX Manager Software version 1.0 (Bio-Rad).

Structural analysis of pollen and stomata
To observe pollen grains via scanning electron microscopy (SEM), anthers were fixed in formalin—acetic acid—alcohol (FAA) for 48 h, dehydrated with increasing concentrations of ethanol (70%, 85%, 95%, and 100%), and fixed on a worktable with double-sided adhesive. The samples were then subjected to CO2 critical point drying, sprayed with gold, and imaged using a Philips XL30 ESEM (Philips Co., Netherlands) at 30 kV [29]. Ten images of each sample were randomly selected, and the size and morphological character of the pollen grains were analyzed. In
order to observe the stomata, leaf samples were cut to 0.5 cm², processed identically to the pollen, and observed via SEM.

Lipid extraction and fatty acid methyl ester analysis

*B. rapa* elongates oleic acid (C18:1) to higher-level monounsaturated fatty acids (MUFAs) through the activity of *FATTY ACID ELONGASE 1 (FAE1)*, which results in a seed oil content of ~41.1% erucic acid (C22:1) and eicosenomic acid (C20:1). However, the canola-type *B. napus* used in the present study possesses a mutated *FAE1*, which results in the near absence of C20 or higher-level MUFAs. Therefore, the F1 hybrid will likely contain about half the amount of higher-level MUFAs between *B. rapa* and transgenic *B. napus*. To confirm this, we analyzed the fatty acid composition of lipids from the seeds of *B. napus*, *B. rapa*, transgenic *B. napus*, and F1 hybrids collected at 10, 20, 30, 40, and 50 DAP.

For lipid extraction, about 100 mg of developing or mature seeds from each plant were crushed in glass tubes with a steel rod, and 0.5 ml toluene and 0.5 ml 5% H₂SO₄ (v/v) in methanol were added to each sample. The tubes were sealed with polytetrafluoroethylene-lined screw caps, and oil extraction and transmethylation were achieved by incubating the samples at 85°C for 90 min, after which 1 ml of 0.9% NaCl (w/v) and 0.5 ml n-hexane were added and the samples were shaken vigorously. The samples were centrifuged at 4000 rpm and room temperature for 2 min. The upper phases, which consisted of fatty acid methyl esters, were transferred to gas chromatograph vials and analyzed, using a GC-2010 plus gas chromatograph (Shimadzu, Kyoto, Japan) with a 30 m × 0.25 mm (i.d.) HP-FFAP column (Agilent Technologies, Santa Clara, CA, USA), while increasing the oven temperature from 190°C to 230°C at 3.5°C/min. Nitrogen was used as the carrier gas, with a flow rate of 1.3 ml/min.

Comparison of *FAE1* sequences

The two paralogous genes, *Bn.FAE1-A8* and *Bn.FAE1-C3*, which are involved in the synthesis of erucic acid and are genetically linked, were cloned from *B. napus*, *B. rapa*, transgenic *B. napus*, and the F1 hybrids and were evaluated for their relevance to the erucic acid content of F1 hybrid seeds.

Genomic DNA was extracted from *B. napus*, *B. rapa*, transgenic *B. napus*, and the F1 hybrid, using the CTAB method [27], and the *FAE1* paralogs were amplified using gene-specific PCR primers (*FAE1-A8* forward: 5′-GGCACCTTTTCATCGGACTAC-3′; *FAE1-A8* reverse: 5′-GATAGAACTCGGGTTTTAGTTG-3′; *FAE1-C3* forward: 5′-GGCACCTTTTCATCGGACTAC-3′; *FAE1-C3* reverse: 5′-TTAACAGAAGATCCTTAACCCC-3′). The PCR reactions contained 50 ng genomic DNA, 4 μl 2.5 mM dNTP mix, 1 U ExTaq polymerase (TaKaRa Bio Inc., Otsu, Japan), 1× ExTaq buffer (TaKaRa Bio Inc.), and 10 pmol of each primer in a total volume of 50 μl. The amplification conditions included an initial denaturation step at 94°C for 4 min; followed by 39 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min; and a final extension step at 72°C for 7 min. Amplifications were performed in a PTC-100 thermal cycler (Bio-Rad).

Results

Outcrossing rate analysis between *Brassica rapa* and transgenic *B. napus*

Hand pollination of 404 *B. rapa* flowers resulted in the formation of 301 pods and, thus, a pod setting ratio of 74.5%, and a mean of 17 seeds were acquired from each pod (*Table 1*). The pod setting ratio of self-pollinated *B. rapa* was 59.4%, and the crossability index was 10.7.
Meanwhile, for self-pollinated *B. napus* and transgenic *B. napus*, the pod setting ratios were 68.6% and 82.8%, respectively, and the crossability indices were 22.1 and 15.2. In addition, all of the herbicide resistant F1 hybrids indicated 100% outcrossing. However, out of the 2,457 F1 hybrid seeds, 600 were cracked (24.4%), owing to precocious germination and seed abortion. Meanwhile, 0.2% and 0% of cracked seeds were observed from self-pollination of *B. napus* and *B. rapa*, respectively.

**Confirmation of hybridization by PCR and flow cytometry**

To study the gene flow from transgenic *B. napus* to the F1 plant, PCR analysis was carried out for all F1 plants showing herbicide resistance (S1 Fig). A 445-bp band that represented the *bar* gene was detected in the PCR amplification products of all of the herbicide resistant F1 hybrids, as was the 738 bp band that represented *BrAGL20*. In addition, the results of flow cytometry indicated that the F1 hybrid (channel 150) was a triploid hybrid, since *B. napus* and transgenic *B. napus* exhibited peaks at channel 200 and *B. rapa* exhibited a peak at channel 100 (S2 Fig).

**Pollen morphology**

To investigate whether the hybridization between *B. rapa* and transgenic *B. napus* would have induced changes in the shape and size of pollens, the pollens of F1 hybrids were compared with both parents, *B. rapa* and transgenic *B. napus* using SEM (Fig 1, Table 2). The length and width of transgenic *B. napus* pollen were less than the pollen of *B. napus*, whereas the F1 pollen was shorter than the parental mean and ~11.8% wider. In addition, about 40% of the F1 pollen grains were smaller average (200 μm²) or exhibited abnormal morphology (Fig 1).

**Correlation between ploidy and stomatal density**

Stomatal apparatus may serve as an important phylogenetic key for plants and thus *B. rapa*, *B. napus*, transgenic *B. napus* and F1 hybrid were compared to identify any differences in the number and form of stomatal apparatus using SEM. The number of stomata on the adaxial and abaxial leaf surfaces was different in each of the plants, and the difference was greatest in *B. rapa* (Fig 2, Table 3).

In *B. rapa*, the number of stomata within 100 μm² surface area was 3.7 on the adaxial surface and 5.7 on the abaxial surface. In *B. napus*, the number of stomata within 100 μm² surface area was 2 on the adaxial surface and 2.7 on the abaxial surface, and in transgenic *B. napus*, the

**Table 1. Outcrossing rate analysis of hybridization (F1) and backcross (BC1) progeny.**

| Plant type | No. pollinated flowers | No. pods | Pod setting ratio (%) | Total no. seeds (no. cracked seeds) | Crossability index (seeds/pod) | Cracked seeds (%) | Outcrossing rate (%) |
|------------|------------------------|----------|-----------------------|------------------------------------|-------------------------------|-------------------|---------------------|
| *Brassica rapa* L. ‘Jangkang’(selfing) | 256 | 152 | 59.4 | 1,629 | 10.7 | 0.7 | - |
| *B. napus* L. ‘Youngsan’ (selfing) | 363 | 250 | 68.8 | 5,526 | 22.1 | 1.0 | - |
| TG.B. napus (selfing) | 337 | 279 | 82.8 | 4,191 (7) | 15.2 | 0.2 | - |
| *B. rapa* ‘Jangkang’♀ x TG *B. napus♂* | F1 | 404 | 301 | 74.5 | 2,457 (600) | 17 | 24.4 | 100 |
| F1 hybrid (selfing) | F2 | 4,053 | - | - | - | - | - |
| *B. rapa* ‘Jangkang’♀ x F1 hybrid♂ | BC1 | 2,294 | 492 | 21.5 | 1,956 (460) | 4 | 23.5 | 50 |

TG *B. napus*, early flowering transgenic *B. napus* L. ‘Youngsan’

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Fig 1. Scanning electron microscope of pollen grains from *Brassica napus*, *B. rapa*, transgenic (TG) *B. napus*, and the F₁ hybrid. Column 1, pollen sacs; column 2, pollen grains; column 3, archopyle; column 4, enlarged image of pollen grains; column 5, pollen grain surface.

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Table 2. Influence of ploidy on floral characteristics of *Brassica napus*, *B. rapa*, transgenic *B. napus*, and F₁ hybrid.

| Ploidy level         | Chromosome no. | Normal pollen | Small pollen |
|----------------------|----------------|---------------|--------------|
|                      |                | Length (μm)   | Width (μm)   |
| *B. napus* 'Youngsan' | 4n             | 45.1 ± 1.1    | 19.1 ± 0.9   |
| *B. rapa* 'Jangkang' | 2n             | 35.7 ± 1.2    | 16.1 ± 0.8   |
| TG *B. napus*        | 4n             | 40.7 ± 0.6    | 17.7 ± 0.7   |
| *B. rapa*♀ × TG *B. napus♂* | 3n   | 37.4 ± 1.7    | 18.9 ± 1.4   |

Values indicate the mean ± standard deviation of three replicates.

a,b,c Alphabet which is different from each other within same column means significantly different (Duncan’s test, p < 0.05).

TG *B. napus*, transgenic *B. napus* L. ‘Youngsan’; *B. rapa*♀ × TG *B. napus♂*, F₁ hybrid of *B. rapa* L. ‘Jangkang’ and TG *B. napus* L. ‘Youngsan’. n, Number of chromosomes in gametic cells.

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number of stomata were 2.3 on the adaxial surface and 3.3 on the abaxial surface. In the F1 hybrid, the number of stomata was 3 on the adaxial surface and 4.3 on the abaxial surface, which was close to the mean of *B. rapa* and transgenic *B. napus*.

Flowering time and expression of flowering-related genes in F1 hybrids
When *B. napus*, *B. rapa*, transgenic *B. napus*, and the F1 hybrid were planted in a greenhouse, transgenic *B. napus* bloomed at 33 d after planting, whereas the F1 hybrid bloomed at 37 d after planting. In addition, under the same conditions, the expression of *BrAGL20* in the apical
meristems of transgenic *B. napus* and the F1 hybrids exhibited peaks at week 4 that were about 4.5 and 2.1 times higher than that of the actin internal control gene, after which they decreased (Fig 3). Similarly, the expression of AGL24 in transgenic *B. napus* and the F1 hybrids increased at week 4, to 3 and 2.1 times that of actin, respectively, after which they decreased. LFY expression also peaked at week 4, to 3.6 and 4.3 times that of actin, in transgenic *B. napus* and the F1 hybrids, respectively, and AP1 expression peaked to 3 and 1.6 times that of actin.

**Fatty acid composition**

The fatty acid composition of *B. napus* seeds was similar to that of transgenic *B. napus* seeds (Fig 4, S1 Table), and at 50 DAP, the fatty acid composition of *B. napus* and transgenic *B. napus* seeds was 6.1% and 5.3% palmitic acid (C16:0), respectively, 3.1% and 2.8% stearic acid (C18:0), 60.7% and 69.4% oleic acid (C18:1), respectively, 19.2% and 14.1% linoleic acid (C18:2), 6.7% and 4.5% linolenic acid (C18:3), 1.5% and 1.4% eicosenoic acid (C20:1), and 0.09% and 0.06% erucic acid (C22:1). At 50 DAP, the fatty acid composition of *B. rapa* seeds was 3.3% C16:1, 1.3% C18:0, 33.3% C18:1, 12.3% C18:2, 5.4% C18:3, 10.8% C20:1, and 30.3% C22:1. At 50 DAP, the fatty acid composition of F1 hybrid seeds was 4.6% C16:0, 1.5% C18:0, 35.9% C18:1, 19.4% C18:2, 6.6% C18:3, 14.4% C20:1, and 17.8% C22:1.

**Characterization of Bn.FAE1-A8 and Bn.FAE1-C3**

Bn.FAE1-A8 from *B. napus* and transgenic *B. napus* exhibited a C-to-T substitution at 845 bp, as well as a deletion of two adenine residues at 1,422–1,423 bp (Fig 5). However, no changes were observed in the *B. rapa* sequences, and the F1 hybrids exhibited a ~50:50 ratio of C and T at 845 bp.

**Seed production of the BC1 generation**

The hand pollination of 2,294 *B. rapa* flowers with F1 hybrid pollen resulted in the formation of 492 pods (21.5% pod setting rate), with an average of four seeds per pod. In the bioassay of BC1 progeny, 50% of BC1 progeny exhibited resistance to 0.3% Basta (outcrossing rate; Table 1), and similar to the F1 hybrid seeds, 460 of 1,956 seeds (23.5%) were cracked. In addition, both the 445 bp band that indicated the amplification of *bar* and the 738 bp band that indicated the amplification of the partial 35S promoter and BrAGL20 were detected in the PCR amplification products of all of the herbicide-resistant BC1 progeny (S1 Fig).

| Ploidy level   | Chromosome no. | No. of stomatal apparatus |
|---------------|---------------|---------------------------|
|               |               | Adaxial                   | Abaxial                   |
| *B. napus* L. ‘Youngsan’ | 4n           | 38                        | *2.0±0.0*                 | 2.7±0.6*                 |
| *B. rapa* L. ‘Jangkang’ | 2n           | 20                        | 3.7±0.6*                 | 5.7±0.6*                 |
| TG *B. napus* | 4n           | 38                        | 2.3±0.6*                 | 3.3±0.6*                 |
| *B. rapa* ♀ × TG *B. napus♂* | 3n           | 29                        | 3.0±0.0*                 | 4.3±0.6*                 |

* Values indicate the mean number of stomatal apparatus ± standard deviation of three replicates.

a,b,c Alphabet which is different from each other within same column means significantly different (Duncan’s test, p < 0.05).

TG *B. napus*, early flowering transgenic *B. napus* L. ‘Youngsan’; *B. rapa* ♀ × TG *B. napus♂*, F1 hybrid between *B. rapa* L. ‘Jangkang’ and TG *B. napus* L. ‘Youngsan’.

Table 3. Influence of ploidy on various floral characteristics in *Brassica napus*, *B. rapa*, transgenic *B. napus* and F1 hybrid.

![Image](36x741 to 143x765)
Outcrossing rate between *B. rapa* and transgenic *B. napus*

Although a number of studies have investigated the gene flow of transgenic *B. napus* to other species in the *Brassicaceae*, the controversy over the environmental impact of GMOs remains. The facts that the wild relative species of *Brassicaceae* easily hybridized with transgenic *B. napus* are greater in number compared to other transgenic crops, and they are largely distributed to the surrounding environment including nearby roadsides to cause environmental issues are the cause of such controversy [14, 1, 19]. However, the acreage of transgenic *B. napus* increases each year, owing to its economic benefits, and the increase is expected to continue.

The hand pollination of a number of *B. rapa* cultivars with transgenic *B. napus* pollen resulted in a wide range of crossability, depending on the *B. rapa* cultivar [12]. In the present study, 17 seeds were harvested per pod, which indicated moderate crossability between the maternal and paternal parents. Variability in the crossability of different *B. rapa* genotypes is
thought to be influenced by the attachment of pollen grains to the stigma, pollen tube penetration, and the abortion of developing embryos [12]. In addition, another study reported that the amount of callose accumulated by papilla cells during pollination between B. rapa and B. napus forms a reproductive barrier that affects crossability [30].

In the present study, the proportion of seeds that were cracked by precocious germination and abortion was 24.5%, which is lower than that observed by Xiao et al. [12]. Cracked seeds

![Graph showing erucic acid content (%) of B. rapa, B. napus, transgenic (TG) B. napus, and F1 hybrid seeds at 10, 20, 30, 40, and 50 days after pollination.](doi:10.1371/journal.pone.0162103.g004)

![Graph showing nucleotide sequence comparison of BnA8.FAE1 and BnC3.FAE1 from Brassica napus, B. rapa, transgenic (TG) B. napus, and F1 hybrids.](doi:10.1371/journal.pone.0162103.g005)
can be attributed to three main causes. When seeds were observed at 10–50 DAP, precocious germination was observed between 10 to 20 DAP when B. rapa was used as the maternal parent and transgenic B. napus was used as the pollen donor (S3 Fig). The phenomenon of precocious germination in hybrids of B. rapa and B. napus been reported previously [12,31,32] and is thought to be caused by seed-specific immunomodulation that switches seed maturation to seed germination [33]. The absence of precocious germination in the hybridization of B. rapa with pollen from resynthesized B. napus suggests that the phenomenon was influenced by the paternal parent [12]. The culling of seeds without normal maturation, owing to the formation of callus tissues during seed development, was another cause of cracking, and although the cause of the phenomenon is still unclear, hormonal imbalance are likely responsible. Lastly, seed abortion, a phenomenon of stalled seed development after fertilization, was considered to be cracked. The formation of cracked seeds was also observed in the backcross of the F1 hybrid to B. rapa, with a cracked seed ratio (23.5%) that was similar to that of the F1 hybrids. The size of F1 hybrid seeds was relatively small compared to that of parent seeds, and the thousand-seed weight was lighter than that of the parents, as well (S2 Table). Small seed size may be a disadvantage for fitness, since it could negatively affect seed emergence, initial seedling size, and initial competitiveness over other plants [34]. The fitness of small seed-derived plants might also be reduced under highly dense or shaded conditions, or in response to drought or herbivory. However, despite the lower thousand-seed weight and seed size of the F1 hybrids in the present study, 100% germination was observed in un-cracked seeds, and the plants also exhibited great vitality.

**Correlation between ploidy level and stomatal density**

Stomatal density, guard cell length, and stomatal plastid number are often used as morphological markers for identifying the ploidy level of plants [35–38]. In the present study, the adaxial and abaxial leaf surfaces of B. napus, B. rapa, transgenic B. napus, and F1 hybrids in the same development phase were examined via SEM. The stomata of the adaxial and abaxial leaf surfaces were different in all four plants, and those of B. napus and transgenic B. napus were also different. The stomata of the F1 hybrid exhibited an intermediate form of B. rapa and transgenic B. napus. In general, as ploidy increases, the density of guard cells and epidermal cells are reported to decrease, whereas the length of the guard cells is reported to increase [37,38]. In the observation of guard cells for diploid and triploid Citrus clementine, there was a positive correlation with ploidy level and stomatal cell length and width and a negative correlation with ploidy level and stomatal density [39]. As the result of analyzing the number of stomata within 100 μm² leaf surface area, the present study found that the number of stomata decreased as ploidy level increased, and the F1 hybrids exhibited intermediate values, when compared to the parent, which is consistent with the data reported by Padoan et al. [39]. The increased guard cell density, which is related to the transpiration rate of guard cells, is required for the movement of water and nutrients [40], and leaf water loss is higher in diploid plants than in triploid plants [39]. Therefore, it remains to be studied that the relationship between the fitness of F1 hybrids and the transpiration rate of their guard cells be analyzed under various weather conditions.

**Expression of flowering-related genes in F1 hybrids**

Transgenic B. napus started flowering on day 33 on average, whereas the F1 hybrids started flowering on day 37. Both the F1 hybrids and the transgenic B. napus bloomed in the greenhouse without vernalization. The difference in flowering time between transgenic B. napus and the F1 hybrids was considered to result from the expression levels of BrAGL20. Transgenic B.
napus, which was homozygous for BrAGL20, exhibited a level of BrAGL20 expression that was ~2.1 times that of the F1 hybrids, which were hemizygous for BrAGL20. This additive transgene expression was also observed in F1 hybrids of wild B. rapa and transgenic B. napus that was homozygous for GFP [41]. The expression level of AGL20 in Arabidopsis is known to play an important role in regulating flowering time, and the gene functions as the key floral activator that integrates several floral inductive pathways [25]. In transgenic B. napus, flowering time is increasingly advanced as the expression level of BrAGL20 increased [26].

Flowering time genes that regulate floral transition have been reported to regulate the activity of floral meristem identity genes, including LFY and AP1(APETALA1) [42–44]. SOC1 regulates LFY expression by directly binding to the LFY promoter, and the LFY protein activates AP1 expression by binding to the cis-element of AP1. However, Lee et al. [45] suggested that heterodimerization of SOC1 and AGL24 is the key mechanism of LFY expression, since LFY expression appears in tissues that express SOC1 and AGL24 simultaneously.

In the present study, the expression of AGL24, LFY, and AP1 was found to increase, along with BrAGL20, in the F1 hybrid and transgenic B. napus from week 4, compared to B. napus and B. rapa. Therefore, the regulation of AGL24, LFY, and AP1 by BrAGL20 expression was likely involved in the early flowering of transgenic B. napus and the F1 hybrids, and the flowering pattern of these plants is thought to have resulted from the additive expression of BrAGL20. The expression peaks of flowering-related genes decreased in the samples from week 5 and week 6.

**Fatty acid composition and FAE1 characterization in F1 hybrids**

The fatty acid composition of Brassicaceae is more genetically diverse than that of other major vegetable oil crops, which suggests that the fatty acid composition can be used as a taxonomic character [46,47]. Among the fatty acids, erucic acid is known to create cardiotoxicity [48]. The B. napus that was developed for edible use has low content of erucic acid, but the B. rapa has erucic acid. Therefore, we aimed to investigate how much content of erucic acid is accumulated in F1 hybrid seeds, assuming when F1 hybrid seeds were released to the environment. In the present study, the fatty acid composition of B. napus, B. rapa, transgenic B. napus, and F1 hybrid seeds was analyzed to determine changes in the fatty acid composition of the F1 hybrid seeds. Erucic acid exhibited the greatest change, and the content of erucic acid in the F1 hybrid seeds was intermediate between B. rapa and transgenic B. napus.

The modern oilseed Brassica napus, which is also called canola, was discovered along with the germplasm of Liho, which is a German spring forage cultivar with low content of erucic acid that has been used as healthy edible oil [49, 50]. The erucic acid synthesis in B. napus seeds is mainly controlled by two genes with additive effect [51], which are the two linked paralogs BnFAE1-A8 and BnFAE1-C3 [52–55]. As reported by Wang et al. [56], the cysteine residue at 845 bp in BnFAE1-A8 is essential for gene function, and its substitution results in loss of function. In addition, the erucic acid content was decreased when by deletions at two sites in BnFAE1-C3: the AA site at 1,422–1,423 bp and the AGGC site at 1368–1371 bp. In the analysis of B. napus varieties, the function of the AA site was found to be more important than the AGGC site [56].

In the present study, BnFAE1-A8 and BnFAE1-C3 were cloned from B. rapa, B. napus, transgenic B. napus, and F1 hybrids, in order to analyze their nucleotide sequences. As a result, we found that cysteine was present at the 845 bp site of BnFAE1-A8 in B. rapa and erucic acid was synthesized at 20 DAP. However, the cysteine residue at the 845 bp site was substituted by a thiamine residue in both B. napus and transgenic B. napus, and the AA site of BnFAE1-C3 was deleted, as well. Although the AGGC site of BnFAE1-C3 remained, the AA site was
apparently more important in the synthesis of erucic acid, as mentioned in the literature [56]. Therefore, no erucic acid was synthesized in transgenic B. napus at 10–50 DAP. Since the BnFAE1-A8 gene in the F1 hybrids was inherited from both B. rapa and transgenic B. napus, the probability of either C or T at the 845 bp site was estimated as 50% each, and sequencing of a number of BnFAE1-A8 from F1 hybrid, confirmed that the probability of the two variations were each 50%.

In addition, the F1 hybrids were verified to contain almost half the level of erucic acid in B. rapa at 30 DAP, which is when erucic acid synthesis began. Thus, the erucic acid level could be used to identify F1 hybrid between B. rapa and transgenic B. napus. In B. rapa, erucic acid was first detected at 20 DAP and increased by over 30% at 30 DAP. In the F1 hybrids, erucic acid was first detected at 20 DAP, gradually increased by about 12.5% at 30 DAP, and then increased to 50% of the total fatty acids after 30 DAP. As for B. napus and transgenic B. napus, erucic acid was not detected in any seeds at 10–50 DAP.

In the present study, the BrAGL20 transgene was transferred to F1 hybrids via hand pollination and was expressed stably, as indicated by early flowering. Even though F2 progeny could not be produced via self-pollination of F1 hybrids, BC1 progeny was obtained by backcrossing the F1 hybrid to B. rapa, which confirms the potential for gene flow in nature if B. rapa is distributed in the vicinity of unintentionally generated co-flowering F1 hybrids. The frequency distribution of chromosome numbers during the meiosis MII stage of sesquidiploid-like F1 hybrids was binomially distributed [57]. Gametes with other chromosomes numbers (from n = 10 to n = 19) failed to exhibit any significant difference in competitive ability before fertilization, but the survival rate after fertilization was reported to differ greatly. BC1 progeny is predicted to have varying numbers of chromosomes and, as a result, to exhibit variation in the expression of the target transgene, as well as in its related phenotypic trait. It is also necessary to backcross each BC1 progeny to generate subsequent progenies and to investigate their environmental sustainability. In addition, the available genetic resources for Brassicaceae are very diverse, and thus, the characteristics of hybrids from GM canola and other Brassicaceae should be investigated, as well.

Supporting Information

S1 Fig. PCR analysis of bar and target gene in Brassica napus, B. rapa, transgenic B. napus, F1 hybrid and BC1 progeny. PC, positive control (DNA of plant expression vector used for transformation); NC, negative control (instead of template DNA DDW was used for PCR); YS, B. napus L. ‘Youngsan’; JK, B. rapa L. ‘Jangkang’; TG, transgenic B. napus L. cv. Youngsan; F1, F1 hybrid of B. rapa L. ‘Jangkang’ and TG B. napus L. ‘Youngsan’; BC1, First backcross generation of B. rapa ♀ and the F1 hybrid ♂.

S2 Fig. Flow cytometry histograms, showing the ploidy levels of Brassica napus, B. rapa, transgenic (TG) B. napus, and F1 hybrids. Red arrows indicate the fluorescent intensity peaks used to determine ploidy levels.

S3 Fig. Seed development of Brassica napus, B. rapa, transgenic (TG) B. napus, and the F1 hybrid at 10, 20, 30, 40, and 50 days after pollination (DAP). The F1 hybrid seeds exhibited precocious germination at 20 days after pollination (DAP) and callus tissues at 40 and 50 DAP.

S1 Table. Fatty acid composition of Brassica napus, B. rapa, transgenic (TG) B. napus, and F1 hybrid seeds. Values indicate the mean fatty acid content (%) ± standard deviation of three
replicates. DAP, days after pollination; B. rapa, B. rapa L. 'Jangkang'; B. napus, B. napus L. 'Youngsan'; TG B. napus, transgenic B. napus L. 'Youngsan'; B. rapa × TG B. napus, F₁ hybrid between B. rapa L. 'Jangkang' and TG B. napus L. 'Youngsan'.

(DOCX)

S2 Table. Seed characteristics of F₁ hybrid between B. rapa and TG B. napus. Values indicate the mean ± standard deviation from three replications. TG B. napus, transgenic B. napus L. cv. Youngsan; B. rapa ♀ × TG B. napus♂, F₁ hybrid between B. rapa L. cv. Jangkang and TG B. napus L. cv. Youngsan.

(DOCX)

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