Diversity of Morphological Traits and Glucosinolate Composition in Backcross Progenies of *Brassica Rapa* L. × *Eruca Vesicaria* (L.) Cav. Hybrids

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Abstract

Breeding at both interspecific and intergeneric levels is an effective method for expanding genetic variation of cruciferous crops; however, few commercially accepted varieties have been released because of difficulties in generating fertile progenies. We employed backcross strategy to introduce *Eruca vesicaria*-specific characteristics into *Brassica rapa* and successfully obtained four BC₁F₁ lines that showed a wide range of diversity of morphological traits and glucosinolate (GSL) composition. Thus, leaf shape, anthocyanin coloration, glucoraphanin content, and red petal veins were found to be inherited mainly from *E. vesicaria*, though most of the morphological characteristics and GSL composition were inherited from intergeneric F₁ plants. Since all BC₁F₁ lines showed post-fertilization barriers, backcrossing with *B. rapa* was performed and BC₂F₁ progenies were successfully obtained, which showed diverse morphological characteristics and GSL composition as well as higher regeneration potential. These results suggest that widening of genetic diversity of *B. rapa* can be achieved through successive reciprocal backcrossing of *B. rapa* × *E. vesicaria* hybrids with *B. rapa*.

1. Introduction

Application of both interspecific and intergeneric crossing in plant breeding has dramatically expanded genetic variation that encompasses functional metabolites, disease resistance, and morphological characteristics that attract consumer and commercial market interests. Spinach mustard (*Brassica rapa* var. *perviridis* L. H. Bailey, a synonym of *B. rapa* L.; AA, 2n = 20), an important leafy vegetable widely cultivated year-round in Japan, includes komatsuna, mizuna, chinese cabbage, and turnip that comprise hundreds of varieties with a narrow genetic and secondary metabolite variation. Therefore, innovative improvement in metabolic components that influence taste and flavor of these vegetables is expected (Velasco et al. 2017; Friedt et al. 2018).

Arugula (*Eruca vesicaria* (L.) Cav.; syn. *E. vesicaria* Mill.; EE, 2n = 22), a delicious leafy vegetable from the Brassicaceae family, was introduced into Japan in 1990s and its sesame-like flavor impressed Japanese consumers (Kim et al. 2004). It contains erucin (4-methylthiobutyl isothiocyanate) and sulforaphane (4-methylsulfinylbutyl isothiocyanate) that belong to the methyl-sulfinyl-type isothiocyanates. Ingestion of erucin and sulforaphane is known to induce phase II detoxifying enzymes that control carcinogenesis in humans (Bell and Wagstaff 2014, 2019). As *B. rapa* does not produce glucosinolates such as erucin and sulforaphane (Ishida et al. 2014; Osada and Aoyagi 2014), interspecific crossing between *B. rapa* and *E. vesicaria* might result in obtaining unique leafy vegetables with functional ingredients and a distinctive sesame-like flavor characteristic to arugula.

Interspecific or intergeneric backcross breeding within the Brassicaceae family is theoretically expected to give three lines in the second generation: an alien gene(s) introgression line (AGIL) as a result of homologous recombination during meiosis, a monosomic alien chromosome-addition line (MAAL) that contains additional heterochromosomes, and a monosomic alien chromosome substitution line (MASL) in which heterogeneous chromosomes are substituted (Kaneko and Bang 2014). Thus, these offspring lines are expected to contain not only various genome composition but also novel, unexpected metabolic pathways acquired through “genomic shuffling.”

Intergeneric crosses between *B. rapa* and *E. vesicaria* were obtained using various combinations of ovule and pollen parents among different varietal groups (Mizushima, 1950a, b; Agnihotri et al. 1990; Matsuzawa et al. 1999). Some of these crossings rendered functional hybrids, whereas most of the intergeneric crosses resulted in no progenies (FitzJohn et al. 2007; Kaneko et al. 2009). Mizushima (1950a, b) successfully performed intergeneric crossing between *B. rapa* as the ovule parent and *E. vesicaria* as the pollen parent to generate haploid and double haploid progenies, though he could not establish stable breeding lines. Recently, we have succeeded in obtaining intergeneric hybrids using the same crossing combination (Kaminishi et al. 2017). Interestingly, the resultant progenies had novel characteristics such as red-blotched petals with red veins as well as a series of glucosinolate (GSL) compounds that both parents produce.

In this study, we employed backcross strategy to introduce *E. vesicaria*-specific characteristics into *B. rapa* using its intergeneric F₁ as a cross parent, and the morphological traits and GSL composition of the progeny were analyzed for genetic variation range.

2. Materials And Methods

2.1. Plant materials, embryo rescue, and growth conditions

Brassica rapa (var. *perviridis*; Misaki series, SAKATA SEED Corp, Yokohama, Japan.) and *Eruca vesicaria* (var. Rucola, KANEKO SEEDS Co., Ltd., Maebashi, Japan) were used as the recurrent and the nonrecurrent parent, respectively, both of which being fixed line, open-pollinated cultivars. Backcrossing was carried out according to Kaneko and Bang (2014) (Fig. 1) using intergeneric hybrids of *B. rapa* and *E. vesicaria* generated in 2017 (Kaminishi et al. 2017).

Siliquae were harvested 35 days after artificial pollination and subjected to embryo culture as follows: siliquae were disinfected with 70% ethanol for 1 min and subsequently immersed in 0.7% sodium hypochlorite for 5 min at room temperature. They were washed with sterilized water twice. Immature embryos were aseptically removed from the siliquae and placed on hormone-free MS medium (Murashige and Skoog 1962), containing 30 g L⁻¹ sucrose and 3 g L⁻¹ gellan gum at pH 5.8. Embryos were then incubated at 25°C under the 16-h light cycle provided by fluorescent bulbs. When the embryos regenerated shoots and became ca. 5 cm high, they were transplanted in plastic pots filled with sterilized clay soil supplemented with N:P:K = 130:700:160 mg L⁻¹ and grown in a growth chamber at 15°C under the 12-h light cycle provided by fluorescent bulbs. As the shoots grew ca. 25 cm high, leaves were harvested for glucosinolate and morphological trait analysis. The plants were then subjected to vernalization by growing in a growth cabinet maintained at 10°C (day) and 5°C (night) under the 10/14-h light regime, respectively, until bolting started. After bolting, plants were returned to a growth chamber and further grown at 15°C under 12-h fluorescent light conditions.

2.2. Pollen tube growth observation

Pollen of *B. rapa* was used to pollinate BC₁F₁ stigmas after emasculation. After 48 h, pistils were fixed with the Farmer's solution and treated 2–3 h with 1 M sodium hydroxide solution. They were subsequently stained with 0.1% aniline blue solution for 30 min to check for callose formation under a fluorescence microscope with UV excitation (Zeiss Axioskop 2 *plus*; Carl Zeiss AG, Oberkochen, Germany). Ten samples were observed per a regenerant.

2.3. Glucosinolate (GSL) analysis

GSL content of regenerated plants was profiled using the method described by Ishida et al. (2011). One leaf was taken from each 25-cm tall plant. Leaves were freeze-dried and ground with mortar and pestle. One millimolar sinigrin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was added to a powdered sample as an internal standard and extraction was performed in 80% methanol at 75°C. The extracts were adsorbed on Sephadex A-25 (Amersham Biosciences AB, Uppsala, Sweden), packed in a mini column, desulfated using sulfatase, and then eluted. GSL content was then analyzed with a HPLC system (LC-20 System; Shimadzu Corporation, Kyoto, Japan) using an Inertsil ODS-2 column (2.1 × 150 mm, GL-Science Co., Ltd., Shinjuku, Japan). The operating conditions were as follows: column temperature 30°C, ultraviolet detection wavelength 229 nm, flow rate 1.5 mL min⁻¹, and sample injection amount 20 µL. The mobile phase consisted of ultra-pure water as the solution A and 20% acetonitrile aqueous solution as the solution B. Initial composition was 99% solution A for the first min, gradually changing to 99% solution B by a linear gradient from the 1st to 21st min. GSL content of broccoli (*B. oleracea* var. *italica*) flower bud, turnip (*B. rapa* subsp. *rapa*), rootstalk, cabbage (*B. oleracea* var. *capitata*), spinach mustard (*B. rapa* var. *perviridis* L.H. Bailey) leaves, arugula (*E. vesicaria* L. Cav) leaves, and rape (*B. napus*) seeds were used to determine types of GSL compounds, according to the correspondence to chromatogram peaks. The amount of each GSL was then calculated using the response factor for sinigrin (Ishida et al. 1995, 2011; Osada and Aoyagi 2014).

2.4. Evaluation of morphological parameters

The following morphological traits were recorded for each regenerated 25 cm tall flowering plant: presence or absence of pungency, leaf sesame-like scent, leaf shape, pubescence, and leaf anthocyanin coloration. Both pubescence and anthocyanin coloration are known to be characteristic for *E. vesicaria* but not for *B. rapa*. In theory, F₁ should be an amphihaploid/diploid and BC₁F₁ sesquidiploids, having at least one pair of the *Eruca* genome. These traits thus were considered “*E. vesicaria*-derived traits.” After bolting, 19 morphological traits were further evaluated: pubescence and anthocyanin coloration of the stalk, flower buds, and pedicel; type of cauline leaves; petal color, shape, size, and vein color; filament color; anthocyanin coloration of the anthers; pollen presence; and type of inflorescence.

2.5. Data analysis

Each of the 19 morphological traits was classified into 2 to 4 grades for data analysis. If a trait was evaluated with two grades, it was classified as either 1 for *B. rapa* type or 2 for *E. vesicaria* type. If a trait was consisted of three grades, it was classified as 1 for *B. rapa* type, 3 for *E. vesicaria* type, and 2 for the intermediate type, and for the traits that were consisted of four grades, they were rated on a scale from 1 to 4, 1 and 4 corresponding to *B. rapa* and *E. vesicaria* types, respectively, and 2 and 3 to intermediate *B. rapa* and *E. vesicaria* types or F₁ type, respectively.

These numerically converted traits were subjected to Spearman's rank correlation analysis using BellCurve for Excel (Social Survey Research Information Co., LTD., Shinjuku, Japan). In cluster analysis, each numerical value was simply replaced with nucleobases: 1 for A, 2 for T, 3 for G, and 4 for C to render a virtual DNA sequence and were then analyzed by maximum parsimony method using MEGA X (Kumar et al. 2018).

3. Results

3.1. Backcross progenies of *B. rapa* × *E. vesicaria* hybrids

Four BC₁F₁ plants (Komeru BC₁ hybrid line) were obtained as backcross progenies from 54 bud pollinations of intergeneric hybrids between *B. rapa* and *E. vesicaria* with the pollen parent, *B. rapa*, Misaki series (Table 1). Each of the four obtained BC₁F₁ plants was further backcrossed with the same pollen donor to obtain the second backcross generation. Total of 2604 bud pollinations resulted in the formation of 534 siliquae, 161 out of which generated embryos suitable for embryo culture. Finally, 30 embryos germinated and 25 out of them developed into straight hybrid plants (Komeru BC₂ hybrid line). Reciprocal crossing between 133 *B. rapa* buds and pollen of BC₁F₁ plants rendered 59 siliquae that generated 203 embryos, 105 out of which germinated and produced 68 reciprocal hybrid plants (MKBC₁ hybrid line). Overall regeneration efficiency of these backcrossed progenies based on the number of plants obtained to that of total buds pollinated was 6.7%, 7.4%, 1.0%, and 51.1% for F₁, BC₁F₁, BC₂F₁ straight cross, and BC₂F₁ reciprocal cross, respectively (Table 1).

Table 1
Results of intergeneric and back crosses between *Brassica rapa* and *Eruca vesicaria*

Cross combination		No. of flowers pollinated	No. of siliques setting	No. of ovules cultured	No. of ovules germinated	No. of ^z plants obtained	Plant ^y yield efficiency
F ₁ generation							
<i>B. rapa</i>	<i>E. vesicaria</i>	15	7	210 ^x	1	1	6.7
BC ₁ F ₁ generation							
Komeru F ₁	<i>B. rapa</i>	54	7	4 ^w	4	4	7.4
BC ₂ F ₁ generation (straight cross)							
Komeru BC ₁ -1	<i>B. rapa</i>	572	84	108 ^w	13	10	1.7
Komeru BC ₁ -2		689	42	23	13	12	1.7
Komeru BC ₁ -3		471	257	16	3	3	0.6
Komeru BC ₁ -4		872	151	14	1	0	0.0
Total		2604	534	161	30	25	1.0
BC ₂ F ₁ generation (reciprocal cross)							
<i>B. rapa</i>	Komeru BC ₁ -1	39	37	141 ^w	70	44	112.8
	Komeru BC ₁ -2	43	3	4	3	2	4.7
	Komeru BC ₁ -3	30	4	4	3	2	6.7
	Komeru BC ₁ -4	21	15	54	29	20	95.2
Total		133	59	203	105	68	51.1
^z Number of individuals that reached the potting stage							
^y (No. of plants obtained / No. of flowers pollinated) × 100							
^x Ovule culture was performed 14 days after the crossing. ^w Embryo culture was performed around 35 days after the crossing							

3.2. Diversity of morphological traits in BC₁F₁ and BC₂F₁ generations

Morphological traits of plants belonging to the BC₁F₁ and BC₂F₁ generations are shown in Fig. 2. *B. rapa* inflorescences had no pubescence or anthocyanin coloration (Fig. 2a), and inflorescences of BC₁F₁ plants, however, had little to no pubescence, similar to those of *B. rapa* (Fig. 2b), whereas those of *E. vesicaria* had a dense pubescence and anthocyanin coloration, especially in the floral buds (Fig. 2c). Anthocyanin coloration of BC₁F₁ plants was observed in both leaf veins and petioles when these plants were grown at 5°C (Fig. 2d). Petals of *B. rapa* were monochromatic yellow, while those of *E. vesicaria* were cream-colored with dark purple veins. BC₁F₁ plants had intermediate-sized yellow petals with red veins and red blotches (Fig. 2e, f). Leaves of *B. rapa* were entire-spatulate-shaped, having neither pubescence nor anthocyanin coloration, leaves of *E. vesicaria* were lyrate-pinnatifid-shaped with visible pubescence and anthocyanin coloration and those of four BC₁F₁ plants had a lyrate-pinnatifid shape (Fig. 2g). Placenta in a silique had one row of ovules in *B. rapa* and two rows of ovules in *E. vesicaria*, whereas that of BC₁F₁ plants had one row of ovules at the distant part and two rows in the middle (Fig. 2h). Cauline leaves wrapped around the stem of *B. rapa* but were unrolled in *E. vesicaria*. Four BC₁F₁ plants had rolled cauline leaves, while one plant had one unrolled cauline leaf and no cauline leaf in *E. vesicaria* (Fig. 2i).

Since efficiency of BC₂F₁ regeneration was very low, presence of a pre-fertilization barrier was presumed. Pollination of ten pistils of four BC₁F₁ plants using *B. rapa* pollen induced callose deposition within pollen tubes that was observed under a fluorescence microscope. Furthermore, pollen tubes of *B. rapa* reached ovules in all four BC₁F₁ plants, indicating that all four BC₁F₁ had no pre-fertilization barrier (Fig. 2j).

BC₂F₁ displayed variation in morphological traits in both backcrosses. Petals of BC₂F₁ were almost of the same size as those of *B. rapa* and very similar to those of BC₁F₁. Petal shape, however, varied greatly: from broadly obovate such as those of *B. rapa*, through obovate, like those of *E. vesicaria*. Petal coloration also varied from monochromatic yellow (yellow petals and veins), through yellow with either red veins and red blotches or orange veins; however, various combinations of these traits were observed (Fig. 2k). In F₁, BC₁F₁, and BC₂F₁ generations, shading of petals with red blotches was associated with red veins only but not with yellow or orange veins. Interestingly, one BC₂F₁ plant had red sectors in its yellow monochromatic petals (Fig. 2l). Although the species belonging to the Brassicaceae family generally have indeterminate inflorescences, some of the BC₂F₁ plants had determinate inflorescences in which corymbs were composed of several flowers (Fig. 2m). Furthermore, although all BC₁F₁ plants had either lyrate or pinnately incised leaves, all the BC₂F₁ plants had various leaf shapes such as entire-spatulate, lyrate-pinnatifid, and lyrate-pinnatifid. Pubescence and anthocyanin coloration of leaves were also observed in some backcrossed plants (Fig. 2n). Examination of chromosomes of pollen mother cells in the first meiotic metaphase of BC₂F₁ revealed the presence of multiple univalent chromosomes in the majority of the plants (Fig. 2o).

Incidence of major morphological traits observed in parental plants and their F₁, BC₁F₁, and BC₂F₁ generations are summarized in Table 2. All the 19 morphological traits that are distinct between *B. rapa* and *E. vesicaria* were classified as either absence or presence, *B. rapa*- or *E. vesicaria*-type, or as a difference in color. When totally 20 straight and 62 reciprocal crosses of BC₂F₁ plants that had grown to the flowering stage were examined, most of the F₁ and BC₁F₁ plants exhibited more similar traits to *E. vesicaria* than to *B. rapa*. On the contrary, the second backcross generation, BC₂F₁, displayed more *B. rapa*-like traits: 5%, 20%, and 75% of the straight crossings had entire leaves, lobed leaves, or incised leaves, respectively, whereas 79% and 9.7% of the reverse crossings had entire and incised leaves, respectively. Leaf margin lobation was entire in *B. rapa*, incised in *E. vesicaria*, and incised in both F₁ and BC₁F₁ generations. *E. vesicaria*-like traits were segregated in reciprocal backcross progenies, suggesting the occurrence of recombination events. No progenies were obtained for pungency taste and the presence of stalk pubescence.

Table 2
Incidence of major morphological traits observed in *Brassica rapa*, *Eruca vesicaria*, their intergeneric hybrids (F₁), and BC₁F₁, BC₂F₁ obtained using straight and reciprocal crosses

Morphological characteristics			<i>B. rapa</i> ^y	<i>E. vesicaria</i> ^x	F ₁	BC ₁ F ₁	BC ₂ F ₁	
(n ²)			(-)	(-)	(1)	(4)	Straight (20)	Reciprocal (62)
Pungency of leaf		absence	100	0	0	25.0	95.0	77.4
		weak	0	0	0	75.0	0	12.9
		strong	0	100	100	0	5.0	9.7
Sesame-like scent of leaf		absence	100	0	0	25.0	85.0	96.8
		weak	0	0	0	75.0	15.0	3.2
		strong	0	100	100	0	0	0
Anthocyanin pigmentation of	leaf	absence	100	0	0	75.0	90.0	96.8
		presence	0	100	100	25.0	10.0	3.2
	stem	absence	100	0	0	75.0	40.0	75.8
		presence	0	100	100	25.0	60.0	24.2
	bud	absence	100	0	0	75.0	95.0	98.4
		presence	0	100	100	52.0	5.0	1.6
	pedicel	absence	100	0	0	0	75.0	93.5
		presence	0	100	100	100	25.0	6.5
Pubescence of	leaf	absence	100	0	0	100	95.0	98.4
		presence	0	100	100	0	5.0	1.6
	stem	absence	100	0	0	0	100	98.4
		presence	0	100	100	100	0	1.6
	bud	absence	100	0	0	75.0	90.0	98.4
		presence	0	100	100	25.0	10.0	1.6
Color of	filament	yellow	100	0	0	0	50.0	91.9
		red	0	100	100	100	50.0	8.1
	anther	yellow	100	0	0	0	65.0	90.3
		red	0	100	100	100	35.0	9.7
Lobation of leaf margin		absence	100	0	0	0	5.0	79.0
		weak	0	0	0	0	20.0	11.3
		strong	0	100	100	100	75.0	9.7
Type of	cauline	<i>B. rapa</i>	100	0	0	75.0	90.0	98.4
		<i>E. vesicaria</i>	0	100	100	25.0	10.0	1.6
	petal	<i>B. rapa</i>	100	0	100	100	40.0	58.1
		<i>E. vesicaria</i>	0	100	0	0	60.0	41.9
Petal	color	yellow	100	0	0	100	90.0	95.2
		lemon	0	0	100	0	10.0	4.8
		cream	0	100	0	0	0	0
	vein color	yellow	100	0	0	0	40.0	85.5
² Number of individuals that reached flowering								
^y Recurrent parent								
^x Nonrecurrent parent								

Morphological characteristics		<i>B. rapa</i> ^y	<i>E. vesicaria</i> ^x	F ₁	BC ₁ F ₁	BC ₂ F ₁	
size	orange	0	0	0	0	10.0	8.1
	red	0	0	100	100	50.0	6.4
	purple	0	100	0	0	0	0
	<i>B. rapa</i>	100	0	0	0	30.0	50.0
	F ₁ /BC ₁ F ₁	0	0	100	100	70.0	50.0
	<i>E. vesicaria</i>	0	100	0	0	0	0
Pollen	absence	0	0	0	0	30.0	11.3
	presence	100	100	100	100	70.0	88.7
Determinate	positive	0	0	0	0	5.0	14.5
	denial	100	100	100	100	95.0	85.5
^z Number of individuals that reached flowering							
^y Recurrent parent							
^x Nonrecurrent parent							

Diversity of the selected 19 morphological traits among the crossing parents *B. rapa*, *E. vesicaria*, and their hybrid and backcross progenies were analyzed in a series by applying the maximum parsimony (Fig. 3). When the breeding parental species were placed at both ends, an F₁ progeny of *B. rapa* × *E. vesicaria*, Komeru F₁, was located next to *E. vesicaria*, while four BC₁F₁ hybrids, Komeru BC₁-1–4, were located next to Komeru F₁. Komeru BC₂ series, the second backcross generation of a straight cross, lay closer to *E. vesicaria* and F₁, whereas MKBC₁ series, a reciprocal cross generation, resided between BC₁F₁ and *B. rapa*. In the reciprocal cross series, 20 BC₂F₁ plants clustered with *B. rapa* and 8 BC₂F₁ plants belonged to another adjacent cluster, indicating that these plants may represent introgression lines bearing alien genes. In addition, six straight cross progenies formed a joint cluster, though, in other BC₂F₁ generations, multiple clusters were formed, regardless if BC₁F₁ individuals were used as parents or reciprocal crossings.

3.3. GSL composition of BC₁F₁ and BC₂F₁ generations

Leaf GSL composition varied between *B. rapa* and *E. vesicaria* plants: *B. rapa* contained glucoiberin, gluconapin, glucoalyssin, glucobrassicinapin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin, while *E. vesicaria* contained glucoerucin, glucoraphanin, glucosativin, and 4-hydroxyglucobrassicin (Supplementary Table 1). Interestingly, all plants belonging to the F₁, BC₁F₁, and BC₂F₁ generations contained all GSLs detected in both parental species but their amounts varied among each generation.

3.4. Correlation between GSL composition and morphological traits of *B. rapa*, *E. vesicaria*, and their hybrid and backcross progenies

Spearman's rank correlation analysis was applied to the analyzed morphological traits and GSL composition of intergeneric crossbred progenies of *B. rapa* and *E. vesicaria*. As shown in Table 3, although the coefficients widely ranged from −0.39 to 0.73, strong positive correlations were recorded between glucosativin and the sesame-like taste (0.73) and intermediate ones between glucoerucin, an *E. vesicaria*-specific GSL, and the sesame-like taste (0.51) and also between glucosativin on one side and pubescence on the stalk (0.53) and anthocyanin pigmentation of buds (0.51), filaments (0.60), and anthers (0.57) on the other side. Interestingly, none of the morphological traits correlated with glucoraphanin.

Table 3 Spearman's rank correlation coefficients for morphological traits and amount of major glucosinolate species in the leaves of *Brassica rapa*, *Eruca vesicaria*, their intergeneric hybrids (F₁), and BC₁F₁, BC₂F₁ backcross straight and reciprocal cross progenies

Morphological characteristics		Glucosinolate species (trivial name)								
		Aliphatic								Indole
		3 carbon side-chains	4 carbon side-chains				5 carbon side-chains			
		Glucoiberin	Glucoerucin	Glucoraphanin	Gluconapin	Progoitrin	Glucosativin	Glucos-alyssin	Glucobrassicinapin	Glucob
Taste	pungent	-0.18	0.08	0.18	0.07	0.07	0.23*	0	-0.08	-0.18
	sesame-like	-0.28**	0.51**	0.19	0.22**	0.11	0.73**	0.31**	-0.06	-0.21*
Leaf shape		-0.18	0.34**	0.25	0	-0.03	0.29**	0.27*	-0.15	-0.29**
Anthocyanin pigmentation of	leaf	-0.29**	0.38**	0.05	-0.03	0.01	0.41**	0.17	-0.14	-0.14
	stalk	-0.03	0.16	0.08	0.02	-0.03	0.17	0.11	-0.08	-0.33**
	bud	-0.1	0.28	0.09	-0.02	-0.09	0.51**	0.08	-0.13	-0.28**
	pedicel	-0.30**	0.23	0.17	0.06	0.05	0.40**	0.05	-0.06	-0.39**
Pubescence of	leaf	0.02	0.21**	0.34**	-0.08	-0.08	0.26*	0.08	-0.14	0
	stalk	-0.35**	0.40**	0.03	0.09	0.17	0.53**	0.30**	-0.07	-0.28**
	bud	-0.16	0.12	0.22*	0.02	0.04	0.18	0.09	-0.03	-0.12
Type of cauline		-0.27	0.27*	0.19	0.01	-0.1	0.47**	0	-0.2	-0.21*
Petal	color	-0.16	0.21*	0.15	-0.17	-0.1	0.30**	0.03	-0.17	-0.1
	vein color	-0.17	0.29**	0.17	0.19	0	0.49**	0.47**	0.01	-0.28**
	size	0.02	0.06	0.08	-0.08	0.03	0.04	0.1	-0.13	-0.31**
	shape	0.11	-0.19	0.21	-0.06	-0.11	-0.12	-0.2	-0.02	0
Color of	filament	-0.21*	0.39**	0.21	0.1	-0.02	0.60**	0.44**	-0.06	-0.30**
	anther	-0.1	0.36**	0.21*	0.16	0	0.57**	0.40**	-0.01	-0.16
Pollen		0.15	0.06	0.09	-0.04	0.09	-0.06	0.01	0	0.02
Determinate		0.06	-0.05	-0.01	0.13	-0.13	-0.13	0.01	0.11	0.15

4. Discussion

Generation of intergeneric hybrids between *B. rapa* (A genome) as the ovule parent and *E. vesicaria* (E genome) as the pollen parent has been previously reported by Mizushima (1950a, b), but further progenies have not been successfully produced either through selfing or by backcrossing thereafter (FitzJohn et al. 2007; Kaneko et al. 2009). In the present study, however, we successfully generated synthetic amphidiploid BC₁F₁ generation and BC₂F₁ generation by backcrossing *B. rapa*. Although the obtained regeneration efficiency was lower than 10% for both BC₁F₁ and the BC₂F₁ straight cross generation as many ovules did not grow and embryo abortion occurred at the time of BC₂F₁ production, BC₂F₁ reciprocal crosses resulted in more than 50% regeneration efficiency and no pre-fertilization barrier in stigmata of BC₁F₁ plants was detected. A low number of embryos that could be used for embryo rescue implies that post-fertilization barriers might exist, since in spite that BC₁F₁ stigmata have actually been pollinated by *B. rapa* pollen to produce BC₂F₁, some ovules died or did not grow enough to make embryos worth rescuing. Kaneko and Bang (2014) previously showed that repeated backcrossing in both interspecific and intergeneric hybrids of Brassicaceae resulted in varying levels of male/female infertility or occurrence of pre-/post-fertilization barriers, depending on the combination of species, and that the overall seed fertility of the hybrids was quite low. Therefore, sesquidiploids would not be easily utilized as ovule parents in intergeneric crossing. Generation of BC₂F₁ plants through backcrossing of sesquidiploid parents, however, would be quite useful for obtaining AGIL, MAAL, and MASL (Kaneko et al. 2009; Kaneko and Bang 2014). Actually, reciprocal crossbreeding of sesquidiploids as pollen parents aided in a successful introduction of a blackleg disease-resistance gene from *B. rapa* to *B. napus* (Yu et al. 2012). Results obtained in the present study also suggest that reciprocal BC₂F₁ crossing may be a promising strategy for introducing genetically stable heterologous alien genes or generating various types of chromosome-introgression lines.

In the present study, the analysis of intergeneric hybrid progeny obtained by crossing between *B. rapa* and *E. vesicaria* showed that morphological traits of *E. vesicaria* were dominantly expressed in the F₁ generation, while some of the *B. rapa*-specific traits phenotypically appeared in the BC₂F₁ generation with various combinations of parents' traits. In addition, this hybridization event generated various plants featured by novel traits not previously found in either

parent such as red or orange veins, absence of pollen, or determinate inflorescences. In particular, red blotches observed in the center of corolla of the F_1 and BC_1F_1 plants represented a novel trait that *Brassica* spp. do not possess. The red-expressing region appears to overlap with the nectar guide area, containing UV-absorbing flavonoids (Sasaki and Takahashi 2002). However, proportions of plants bearing these traits differed greatly between the straight and reciprocal hybridizations. An increased diversity of morphological traits was recorded in interspecific hybrid progenies of *B. oleracea* var. *alboglabra* and *B. rapa* var. *purpurea* in the shape and color of inflorescences, leaves, stalks, and several other characteristics (Zhang et al. 2016). Furthermore, in the BC_2F_1 population derived from a cross between *B. napus* with yellow flowers and *Orychophragmus violaceus* with large purple flowers, a unique individual, having yellow flowering petals, while being red before opening, was obtained (Ding et al. 2013). In alloplasmic F_7 (BC_1F_6) progenies of a cross between *E. vesicaria* and *B. rapa*, plants whose stamens were changed into petals were recorded (Matsuzawa et al. 1999). In the present study, however, no such plants were observed.

In this study, all the four BC_1F_1 plants showed a similar phenotypic appearance in both petal characteristics and leaf shape as the F_1 ovule parent. Because morphological traits of F_1 (AAEE) and BC_1F_1 (AAE) are considered to result from the addition of the E genome, both alien chromosome-addition lines are expected to have similar phenotypes, while BC_2F_1 displayed not only characteristics specific to their parents but also novel characteristics such as yellow flower vein color. Mizushima (1950a) reported up to eight divalent chromosomes during metaphase of F_1 (AE) hybrids, which indicates that the A and E genomes can pair, implying occurrence of heterozygote translocation between chromosomes of these two genomes in the hybrid progeny. A dynamic chromosomal structural modification due to polyvalent chromosome formation could occur not only in E-derived gene expression but also in A-genome gene expression. These genomic alterations may trigger interactions among newly introduced or rearranged genes, which might result in the emergence of novel phenotypes.

Among 4-carbon side-chains, glucoerucin, glucoraphanin, gluconapin, and progoitrin that are produced in the biosynthetic pathway of aliphatic GSLs, gluconapin was the major GSL found in *B. rapa*. By contrast, *E. vesicaria* contained glucoerucin, glucoraphanin, and glucosativin that are synthesized from glucoerucin (Bennett et al. 2002; Bell and Wagstaff 2014, 2019; Ishida et al. 2014; Osada and Aoyagi 2014; Fechner et al. 2018). Both F_1 and BC_1F_1 hybrids obtained in the present study contained GSLs synthesized by both parental species. If these hybrids contained the *E. vesicaria* genome, compounds produced in the downstream part of the biosynthetic pathway would also accumulate. As several factors that control the GSL biosynthesis have been elucidated (Ishida et al. 2014), it is highly plausible that expression patterns of transcription factors related to the biosynthetic pathway of aliphatic GSLs in *Brassica* species that possess alien chromosomes may be regulated.

Some morphological traits of *E. vesicaria* were dominantly inherited in the BC_2F_1 population that was derived from an intergeneric hybridization between *B. rapa* and *E. vesicaria*. The relationship between morphological traits and GSL composition observed in this study revealed that sesame-like taste was strongly correlated with glucosativin but weakly correlated with glucoerucin, suggesting that sesame flavor may be related to the biosynthetic pathways of these compounds. This supports the fact that glucosativin itself is a typical flavor agent of crushed fresh arugula leaves (Bennett et al. 2002; Fechner et al. 2018; Raffo et al. 2018; Bell and Wagstaff 2019). In a previously reported sensory analysis, *E. vesicaria* accessions with a broad range of GSL profiles, glucosativin, and its dimer did not correlate with bitterness, while glucosativin was positively correlated with an earthy flavor and its dimer was positioned between earthy and pungent attributes, but it was not significantly correlated with any specific sensation (Bell et al. 2017). However, the authors did not mention “sesame-like taste or flavor.” Thus, it would be difficult to determine a specific GSL profile from particular morphological traits expressed in the BC_2F_1 generation.

In the present study, we successfully generated intergeneric progenies from a cross between *B. rapa* and *E. vesicaria* up to the BC_2F_1 generation. Although BC_2F_1 progenies exhibited few characteristics of *E. vesicaria*, new traits such as red blotches and red and orange veins in petals as well as some characteristic *E. vesicaria* GSLs could be introduced into the *B. rapa* gene pool. These findings demonstrate that repeated backcrossing can be a promising method to introduce useful and/or unique alien traits into closely related species after obtaining intergeneric cross hybrids.

Declarations

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Author Contribution Statement

Aiko Kaminishi: developed the plants, conducted the experiments, and analyzed the data. Fumika Miyohashi: partly participated in the experiments. Nobuhiro Kita: conceived the project, designed the research, and critically reviewed and improved the final manuscript.

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Figures

Figure 1

Backcrossing scheme for the hybridization between *Brassica rapa* and *Eruca vesicaria*. Structure of each genome, including progenies and the number of hybrid lines obtained are shown in parenthesis and at the figure bottom

Figure 2

Morphological traits of flowers, buds, petals, habitus, leaves, pods, and chromosomes of intergeneric hybrid BC1F1 and BC2F1 plants obtained from backcrossing with *Brassica rapa* and *Eruca vesicaria*. Inflorescences of *B. rapa* (a), Komeru BC1-2 hybrid (b), and *E. vesicaria* (c); habitus of Komeru BC1-1 hybrid (d); corolla (e), petal (f), leaf shape (g), placenta (h), and proximal end of a cauline leaf (i) of *B. rapa* (left), four intergeneric BC1F1 hybrids (center), and *E. vesicaria* (right) (note that the second plant from left of four intergeneric BC1F1 hybrid plants has an unrolled cauline leaf); pollen tube growth toward ovules of an intergeneric hybrid BC1F1 (j); corollas of *B. rapa* (left), four BC2F1 hybrids (k), and of another BC2F1 plant(l); determinate inflorescence of a BC2F1 plant (m); leaf shapes of six BC2F1 hybrids (n); and chromosome behavior in metaphase I of a BC2F1 hybrid (o)

Figure 3

Cladogram of totally 89 plants of *Brassica rapa*, *Eruca vesicaria*, and their intergeneric hybrid progenies based on taste and morphological traits and constructed using the maximum parsimony algorithm. Each morphological trait was classified into two to four grades, according to its presence/absence, type, or level and was replaced with nucleobase letters A, G, T or C to mimic nucleotide sequences (Kumar et al. 2018). Recurrent parent, *B. rapa*; nonrecurrent parent, *E. vesicaria*; F1, Komeru F1 (red); BC1F1, Komeru BC1 (purple); straight cross BC2F1, Komeru BC2 (green); reciprocal cross BC2F1, MKBC1 (blue)

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