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ORIGINAL PAPER

Genetic analysis of hybrid seed formation ability of *Brassica rapa* in intergeneric crossings with *Raphanus sativus*

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Abstract A hybridization barrier leads to the inability of seed formation after intergeneric crossings between *Brassica rapa* and *Raphanus sativus*. Most *B. rapa* lines cannot set intergeneric hybrid seeds because of embryo breakdown, but a *B. rapa* line obtained from turnip cultivar 'Shogoin-kabu' is able to produce a large number of hybrid seeds as a maternal parent by crossings with *R. sativus*. In 'Shogoin-kabu' crossed with *R. sativus*, developments of embryos and endosperms were slower than those in intraspecific crossings, but some of them grew to mature seeds without embryo breakdown. Intergeneric hybrid seeds were obtained in a 'Shogoin-kabu' line at a rate of 0.13 per pollinated flower, while no hybrid seeds were obtained in a line developed from Chinese cabbage cultivar 'Chiifu'. F_1 hybrid plants between the lines of 'Shogoin-kabu' and 'Chiifu' set a larger number of hybrid seeds per flower, 0.68, than both the parental lines. Quantitative trait loci (QTLs) for hybrid seed formation were analyzed after intergeneric crossings using two different F_2 populations derived from the F_1 hybrids, and three QTLs with significant logarithm of odds scores were detected. Among them, two QTLs, i.e., one in linkage group A10 and the other in linkage group A01, were detected in both the F_2 populations. These two QTLs had contrary effects on the number of hybrid seeds. Epistatic interaction between these two QTLs was revealed. Possible candidate genes controlling hybrid seed formation ability in QTL regions were inferred using the published *B. rapa* genome sequences.

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Introduction

Interspecific and intergeneric hybridizations are important means of breeding for developing crop cultivars having novel genetic traits, which cannot be introduced into cultivars by conventional intraspecifc crossings, and have been performed using many crop species. However, hybrid production by interspecific and intergeneric crossings is hampered by reproductive isolation due to hybridization barriers. The hybridization barrier contributes to speciation and evolution of organisms by inhibiting gene flow from one species to other species.

The hybridization barrier is classified into two types: a pre-zygotic barrier and a post-zygotic barrier. The pre-zygotic barrier includes interspecific incompatibility (Udagawa et al. 2010), incongruity (de Nettancourt 2001), and inability of fertilization caused by abnormality of pollen guidance (Dresselhaus and Márton 2009), and the post-zygotic barrier includes hybrid embryo breakdown, hybrid abnormality, and hybrid sterility (Lowry et al. 2008). As genetic loci responsible for interspecific incompatibility between maize and teosinte, *Ga1, Ga2* and *Tcb1* have been reported (Evans and Kermicle 2001; Kermicle and Evans 2010). A quantitative trait locus (QTL) participating in interspecific incompatibility of the stigmas of *Brassica rapa* with pollen of *Brassica oleracea* has been analyzed

(Udagawa et al. 2010). In the post-zygotic barrier, hybrid seed abortion is observed in many plant species after interspecific and intergeneric crossings (Sharma and Gill 1983; Bushell et al. 2003). In many cases, aberrant endosperm development has been observed together with embryo breakdown, suggesting that endosperm abnormality is related to the embryo breakdown. Balance of ploidy levels between paternal and maternal parents has also been reported to be important for successful embryo development by interspecific crossings in *Solanum* (Johnston and Hanneman 1982), *Avena* (Nishiyama and Yabuno 1978), and *Arabidopsis* (Bushell et al. 2003; Jossefson et al. 2006). As mechanisms of aberrant development of endosperm, various hypotheses such as Endosperm Balance Number (EBN) (Johnston et al. 1980) and Polar Nuclei Activation (PNA) (Nishiyama and Yabuno 1978) have been proposed. These hypotheses have been explained by genomic imprinting in endosperm (Kinoshita 2007).

Since the genus *Brassica* includes many important crop species, such as oilseed rape, mustard, leafy vegetables, and root crops, *Brassica* breeding using intergeneric crossings with *Raphanus sativus* (Karpechenko 1924), *Moricandia arvensis* (Takahata and Takeda 1990), *Sinapis alba* (Brown et al. 1997), and *Crambe abyssinica* (Wang and Luo 1998) has been carried out. Among the species used for intergeneric crossings, *R. sativus* is useful as a gene source for disease resistance, e.g., resistance to clubroot (Akaba et al. 2009), and for producing cytoplasmic male sterile lines (Koizuka et al. 2003). Because hybrid seed abortion is commonly observed in intergeneric crossings between *B. rapa* as a maternal parent and *R. sativus* as a paternal parent, embryo rescue by embryo culture or ovary culture is essential for producing hybrid plants. However, hybrids between *Brassia* and *Raphanus* have been reported to be obtainable without embryo rescue in some combinations of cultivars or lines (Karpechenko 1924). Elucidation of molecular mechanisms controlling differences of intergeneric hybrid formation abilities in *B. rapa* lines may provide a means to overcome the hybridization barrier.

We have previously revealed a line obtained from turnip cultivar 'Shogoin-kabu' to have high ability to set seeds of intergeneric hybrids between *B. rapa* and *R. sativus* (Kaneko et al. 1993). This line is hypothesized to have an allele of a gene enabling efficient seed production of intergeneric hybrids. In the present study, we developed single nucleotide polymorphism (SNP) markers for construction of a linkage map using an F_2 population obtained from a cross between a 'Shogoin-kabu' line and an inbred line of Chinese cabbage cultivar 'Chiifu', and analyzed QTLs for efficiency of seed production of intergeneric hybrids. Candidates of the genes responsible for hybrid formation ability were screened from putative genes in the QTL regions using the published *B. rapa* genome sequences (Wang et al. 2011).

Materials and methods

Plant materials

A line obtained by selfing of turnip cultivar 'Shogoin-kabu' in *Brassica rapa* ($2n = 20$), which can yield a few hybrid seeds by intergeneric crossings with *Raphanus sativus* 'Shogoin-daikon' ($2n = 18$), and an inbred line of Chinese cabbage cultivar 'Chiifu' in *B. rapa*, which cannot set hybrid seeds by intergeneric crossings, were used as parents of F_2 populations. These lines have been developed at the laboratory of plant breeding, Utsunomiya University, Japan. Two subsets of 130 and 145 F_2 plants were used in 2008 and 2010, respectively. The F_2 plants and their parental lines were cultivated using 24-cm pots in an unheated greenhouse at Utsunomiya University in 2008 and in such a greenhouse at Tohoku University, Japan, in 2010. *R. sativus* 'Shogoin-daikon' was used as a paternal parent of intergeneric crossings.

Observation of pre- and post-zygotic barriers

To observe pollen tube growth after pollination, flower buds 2 days before anthesis and open flowers just after anthesis of the maternal parents were emasculated and pollinated with fresh pollen of *R. sativus*. Forty-eight hours after pollination, 20 pistils were observed under a fluorescent microscope after staining with 0.1 % aniline blue in 2 % K_3PO_4. For investigating embryo development after intergeneric crossings, ovaries developed from bud-pollinated pistils 20 to 25 days after pollination (DAP) were fixed by FAA (formaldehyde:acetic acid:50 % ethanol = 5:5 :90), dehydrated by ethanol series (30, 50, 70, 85, 95, 100 and again 100 %) for 1 h each. After dehydration, ethanol was replaced with solvents of 75 % ethanol with 25 % xylene, 50 % ethanol with 50 % xylene, 25 % ethanol with 75 % xylene, and 100 % xylene. By increasing the concentration of paraffin in xylene, the ovaries were embedded in paraffin and sectioned by a microtome.

Investigation of hybrid seed formation in intergeneric crossings

To evaluate hybrid seed formation ability in intergeneric crossings, about 100 pistils per plant of *B. rapa* were crossed with pollen of *R. sativus* 'Shogoin-daikon' by bud-pollination 2 days before anthesis, and the number of

hybrid seeds per pollinated pistil was counted. Since there is a possibility that selfed seeds are mingled with hybrid seeds obtained by intergeneric crossings, all harvested seeds were sown and intergeneric hybrid plants were screened by morphological observation (leaf shape, flower color; Supplementary Fig. 1) and analyses with species-specific internal transcribed spacer 1 (ITS1) probes (Tonosaki and Nishio 2010) to investigate the number of true hybrid seeds.

Preparation of genomic DNA and development of SNP markers

Genomic DNA was isolated by the modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). A 0.1 g piece of a leaf was pulverized in liquid nitrogen and suspended in 2× CTAB solution (2 % CTAB, 100 mM Tris–HCl buffer, pH 8.0; 1.4 M NaCl; 20 mM ethylenediaminetetraacetic acid). After chloroform/isoamyl alcohol (24:1) extraction, DNA was precipitated by the addition of isopropanol. DNA was dissolved in 1× TE buffer and treated with RNase.

SNP markers showing polymorphism between the 'Shogoin-kabu' line and the 'Chiifu' line, which were the parents of F_2 plants used for QTL analysis, were screened from the SNP markers developed by Li et al. (2009) and Udagawa et al. (2010). For developing new SNP markers, we used primer pairs designed by Li et al. (2011) and those designed from bacterial artificial chromosome (BAC) sequences of *B. rapa* published by the Multinational *Brassica* Genome Project (http://www.brassica.info/) in polymerase chain reaction (PCR). Single DNA fragments amplified by PCR were directly sequenced for sequence comparison between parental lines to identify SNPs. Bridge probes were designed for developing dot-blot SNP markers according to Shiokai et al. (2010). SNPs of F_2 plants were analyzed by the method of dot-blot SNP analysis (Shirasawa et al. 2006).

Construction of a linkage map and analyses of QTLs and epistasis

Construction of a linkage map was performed using JoinMap ver. 4.0 software (van Ooijen 2006). Linkage groups were identified by the threshold value of 0.3, and the Kosambi mapping function was used to convert recombination frequencies into map distances (cM). QTL analysis was performed using composite interval mapping (CIM) analysis with Windows QTL Cartographer v2.5. A permutation test was applied to each data set (1,000 repetitions) to determine the LOD thresholds ($P = 0.05$). Epistatic interaction between pairs of QTLs was tested using two-way ANOVA, as represented by the nearest marker loci in the F_2 populations.

Sequence and expression analyses of candidate genes

Based on sequence comparison of QTL regions of the *B. rapa* genome, two candidate genes were selected. The published nucleotide sequences of these genes, i.e., *Bra023933* and *Bra002667* (Wang et al. 2011), were used for designing primers for nucleotide sequencing.

For RNA extraction, ovules were collected at 15 days after intraspecific crossings in a greenhouse at 20 °C. Total RNA was extracted from 30 mg of ovules using the SV Total RNA Isolation System (Promega Corp.), and first strand cDNA was synthesized from 1 μg of total RNA by reverse transcription using a superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). RT-PCR was performed by specific primers of *Bra023933* (forward primer: 5′-TACACTGTAAGCTGGGCGCG-3′; reverse primer: 5′-TCCCAGTTTCAACGTTCCAC-3′) and *Bra002667* (forward primer: 5′-GCTTATTGTTTGGGACGTTAGC-3′ and reverse primer: 5′-TCTGAAACTTTGCTGGTGT-GAC-3′), by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min.

Results

Seed formation of intergeneric hybrids between *B. rapa* and *R. sativus* and microscopic observation of pistils after intergeneric crossings

Two *B. rapa* lines, i.e., a 'Shogoin-kabu' line and a 'Chiifu' line, were crossed as female parents with *R. sativus* cultivar 'Shogoin-daikon' as the male parent. After maturity, siliques were harvested and analyzed for hybrid ovule size, seed size, and seed shape for comparison with intraspecific crossings. In intraspecific crossings, most ovules developed to big plump seeds (seed size 2.69 ± 0.32 cm^2) in siliques 15 days after pollination (Fig. 1a, d) and the seed number per pollinated flower was about 20. In intergeneric crossings of a 'Chiifu' line and *R. sativus*, no expanded ovules were observed in siliques 15 days after pollination (Fig. 1b) and only small-shriveled seeds were obtained (Fig. 1e). On the other hand, in intergeneric crossings of a 'Shogoin-kabu' line and *R. sativus*, most ovules were enlarged, and some of them developed into small seeds about 1 mm in diameter. Two types of seeds were obtained: small shriveled seeds and small plump seeds (seed size 1.19 ± 0.32 cm^2) (Fig. 1c, f). All the small shriveled seeds obtained in these two lines lacked germination ability, while all the small plump seeds obtained in the 'Shogoin-kabu' line germinated. All the

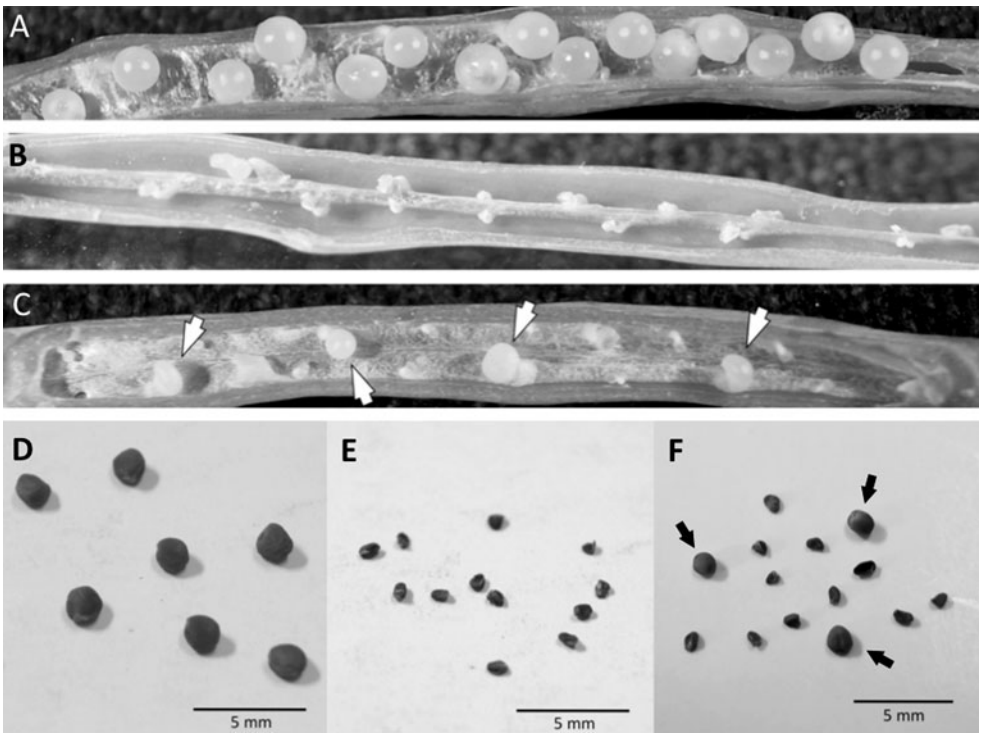

Fig. 1 Immature and mature seeds obtained by intraspecific and intergeneric crossings. Seeds obtained by intraspecific crossings of 'Chiifu' (**a, d**) and intergeneric crossings of 'Chiifu' (**b, e**) and 'Shogoin-kabu' (**c, f**) with *R. sativus* pollen. **a–c** immature seeds at 15 DAP. **d–f** mature seeds at 45 DAP. *Arrows* in **c** and **f** indicate hybrid seeds

plants from the small plump seeds showed leaf morphology and flower color of intergeneric hybrids between *B. rapa* and *R. sativus* (Supplementary Fig. 1), and those analysed with the species-specific ITS1 probes were revealed to be intergeneric hybrids.

To investigate the pre-zygotic barrier, pollen grains of *R. sativus* 'Shogoin-daikon' were pollinated onto the stigmas of open flowers or buds 2 or 3 days before anthesis of a 'Shogoin-kabu' line and a 'Chiifu' line, and pollen tube behavior was observed. In all the plants of both parental lines, pollen tubes grew into micropyles. The numbers of pollen tubes in the styles of pollinated buds were more than those of open flowers, suggesting the presence of intergeneric incompatibility between these *B. rapa* parental lines and *R. sativus* 'Shogoin-daikon'. The numbers of pollen tubes in the styles were not different between the 'Shogoin-kabu' line and the 'Chiifu' line, indicating comparable levels of intergeneric incompatibility between these *B. rapa* parental lines.

For investigating the post-zygotic barrier, the development of embryos and that of endosperms were observed under a microscope after intraspecific and intergeneric crossings to the stigmas of buds 2 or 3 days before anthesis. At 12 DAP of intraspecific crossings, embryos were at the globular embryo stage, and endosperm nuclei (EN), a micropylar endosperm (MCE), and a chalazal endosperm (CZE) were observed around a central vacuole (CV) (Fig. 2a). Ovaries grew to about 1.9 mm at 16 DAP and torpedo-shaped embryos were observed. The endosperms were cellularized in embryo sacs as reported by Nishiyama and Inomata (1966) (Fig. 2b). At 20 DAP, embryos developed to the walking stick stage (Fig. 2c). On the other hand, in intergeneric crossings of the 'Chiifu' line, ovaries were small, about 1.5 mm, even at 20 DAP, and embryo sacs were shrunken. No developed embryos and endosperms were observed (Fig. 2d). In the 'Shogoin-kabu' line, most ovaries were small and shriveled after intergeneric crossings as in the 'Chiifu' line, but in some small plump ovaries, ca. 1.6 mm, development of embryos to the heart stage or the torpedo stage was observed at 20 DAP (Fig. 2e, f). In these ovaries with developed embryos at 20 DAP, EN were observed, but endosperms were not cellularized. A shape of CZE was different from that of intraspecific crossings. At 25 DAP, embryos remained at the heart stage and no cellularization of endosperms was observed in some small-plump ovaries with expanded embryo sacs (Fig. 2g), while endosperms were cellularized and embryos developed to the walking stick stage in other small-plump ovaries with shrunk embryo sacs (Fig. 2h).

Phenotypic variation of the hybrid seed formation ability

Ten plants each of the 'Shogoin-kabu' line, the 'Chiifu' line, and their F_1 hybrid were examined, and 100 flowers

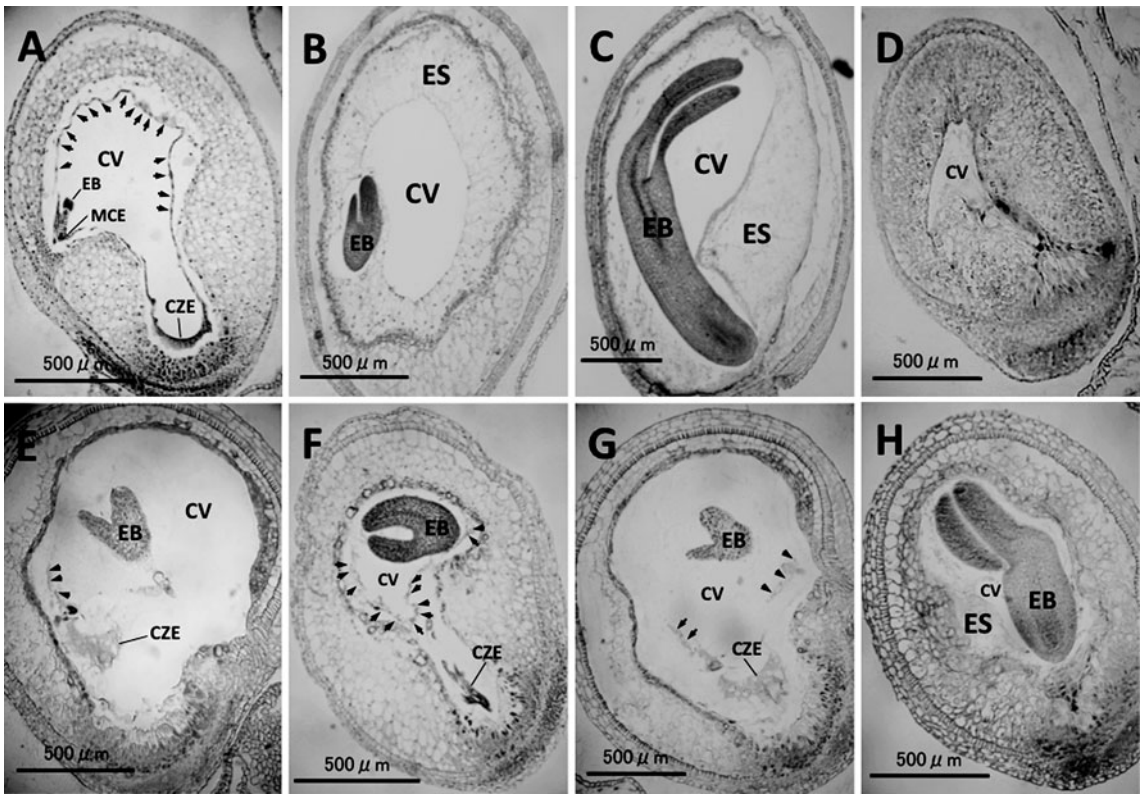

Fig. 2 Ovule development after intraspecific and intergeneric crossings Ovules 12 (**a**), 16 (**b**), and 20 (**c**) days after intraspecific crossings, an ovule of 'Chiifu' 20 days after intergeneric crossing with *R. sativus* (**d**), and ovules of 'Shogoin-kabu' 20 (**e, f**) and 25 (**g, h**) days after intergeneric crossings with *R. sativus* are shown. *Arrows* indicate endosperm nuclei (EN). *EB* embryo, *ES* endosperm, *CV* central vacuole, *MCE* mycropylar endosperm, *CZE* chalazal endosperm

per plant were pollinated with *R. sativus* pollen. The average number of hybrid seeds per pollinated flower was 0.13 ± 0.01 in the 'Shogoin-kabu' line, while no seed was obtained in the 'Chiifu' line. F_1 hybrid plants set 0.68 ± 0.35 hybrid seeds per flower on average, more than those of both the parental lines. One hundred thirty F_2 plants grown in 2008 set 3,587 hybrid seeds in total and 0.38 ± 0.52 per flower on average (Supplementary Fig. 2a). The 145 F_2 plants grown in 2010 set 0.53 ± 0.67 per flower on average (Supplementary Fig. 2b). The number of hybrid seeds per pollinated flower in the F_2 plants was distributed continuously, suggesting that the ability to form intergeneric hybrid seeds is a quantitative trait.

DNA marker production

DNA markers showing polymorphism between the 'Shogoin-kabu' line and the 'Chiifu' line were selected. Only 27 of 212 SNP markers developed by Li et al. (2009) and Udagawa et al. (2010) showed polymorphism between these two lines. Forty-nine primer pairs of the SNP markers showing no polymorphism were used for amplification of genomic DNAs of these two lines, and amplified DNA fragments were sequenced. SNPs were identified in twelve PCR products, and eight SNP markers were produced from them. By PCR, 587 primer pairs in 2,592 tested pairs designed by Li et al. (2011) amplified single DNA fragments from both the parental lines. Nucleotide sequences of PCR products amplified from these lines by the 587 primer pairs were determined. Comparison of sequences between these two lines revealed 165 PCR fragments to have SNPs, and 129 of them were used for developing dot-blot SNP markers. Among 67 primer pairs designed from the published sequences of BAC clones of *B. rapa,* 43 primer pairs yielded single DNA fragments from both the parental lines by PCR. Nucleotide sequencing of these fragments identified SNPs in 40 DNA fragments, ten of them being used for producing dot-blot SNP markers. In total, 174 dot-blot-SNP markers showing polymorphism between the 'Shogoin-kabu' line and the 'Chiifu' line were obtained.

Construction of a linkage map and QTL analysis

The total 174 SNP markers were used for genotyping of the 130 F_2 plants grown in 2008, and linkage of these markers was analyzed. Among them, 148 markers were mapped in

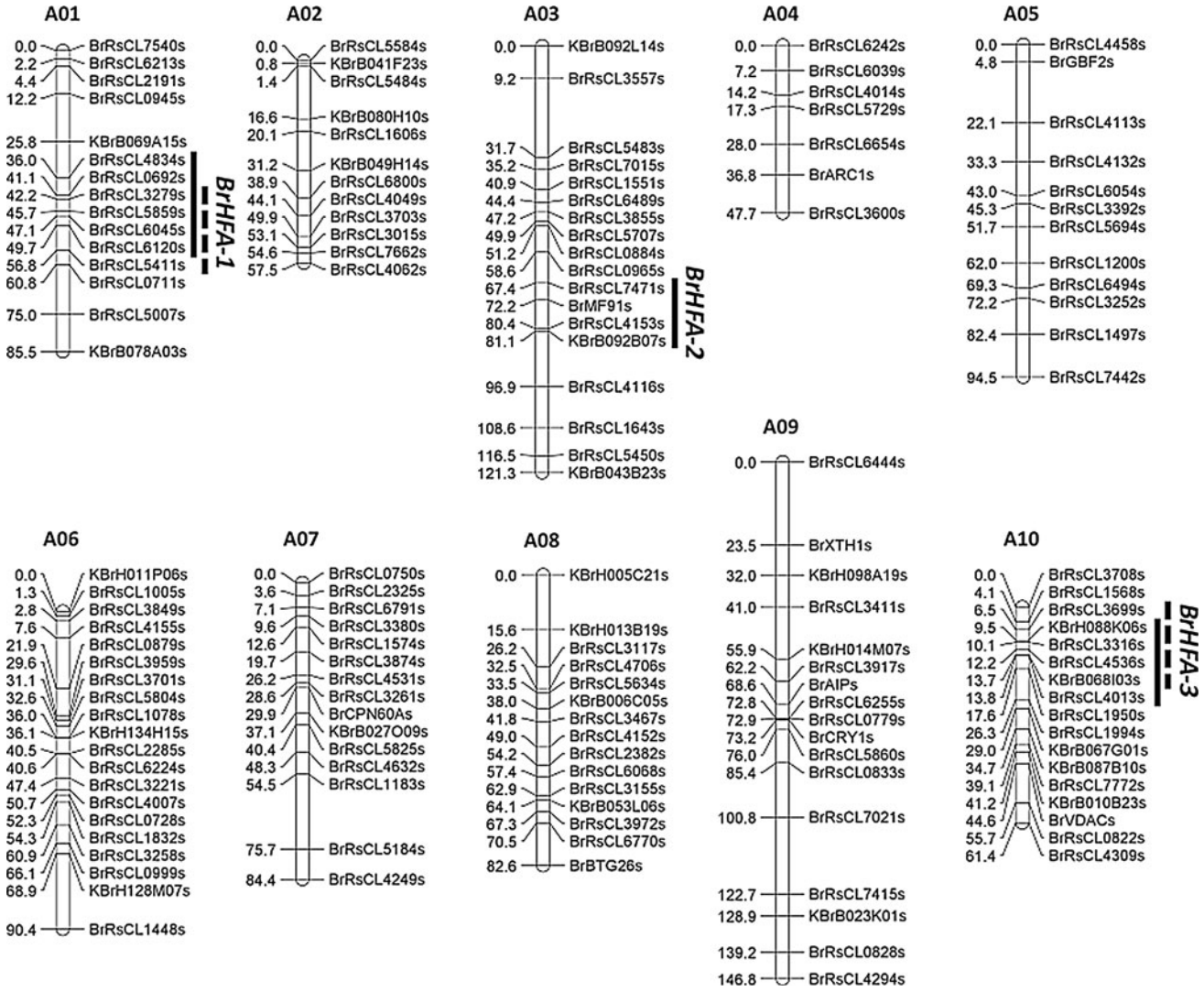

Fig. 3 Linkage map of SNP markers in *B. rapa* and QTLs for intergeneric hybrid seed formation. *Solid bars* indicate QTL regions detected in 2008, and *broken line bars* indicate QTL regions detected in 2010

ten linkage groups, and the other 23 markers were not mapped(Fig. 3, Supplemental Table 1). This linkage map covered 871.8 cM, the average distance between nearest markers being 5.8 cM. The longest gap between the markers was 23.5 cM. These ten linkage groups were assigned to the published linkage groups, i.e., A01 to A10, based on sequence identity of the SNP markers with the published genomic sequence data (Multinational Brassica Genome Project 2011).

Three QTLs were detected on A01, A03, and A10 by QTL analysis using the data on the number of hybrid seeds per pollinated flower in the 130 F_2 plants grown in 2008 (Fig. 3), and named *qBrHFA-1. qBrHFA-2,* and *qBrHFA-3,* respectively (Table 1). *qBrHFA-1* on A01 had a LOD score of 6.05 and an explained phenotypic variance of 21 %. Although *qBrHFA-1* had the highest effect among the three QTLs, its additive effect was −0.27, indicating that a 'Shogoin-kabu' allele on this QTL reduces hybrid seeds. *qBrHFA-3* on A10 had an LOD score of 4.7 and a positive additive effect of 0.28 with an explained phenotypic variance of 15 %. These two QTLs were again detected in the analysis using the 145 F_2 plants in 2010, while LOD score of *qBrHFA-2* on A03 did not exceed the threshold value, 2.5. In 2010, *qBrHFA-3* had a higher LOD score, a greater additive effect, and a greater explained phenotypic variance than *qBrHFA-1*.

Analysis of epistasis between *qBrHFA-1* and *qBrHFA-3*

The hybrid formation ability of the F_1 hybrids between the 'Shogoin-kabu' line and the 'Chiifu' line was higher than both the parental lines. The existence of epistasis between two QTLs for hybrid formation ability can be hypothesized.

Table 1 QTLs for hybrid seed formation ability detected in F_2 populations obtained from a cross between 'Shogoin-kabu' and 'Chiifu'

	LG	Marker interval	LOD	AE[a]	DE[b]	PVE (%)[c]
2008						
qBrHFA-1	A01	BrRsCL4834s–BrRsCL6120s	6.05	−0.27	0.25	21
qBrHFA-2	A03	BrRsCL7471s–KBrB092B07s	3.5	0.21	0.07	5.9
qBrHFA-3	A10	KBrH088K06s–BrRsCL4013s	3.8	0.28	0.1	15
2010						
qBrHFA-1	A01	BrRsCL3279s–BrRsCL5411s	3.5	−0.14	0.5	12
qBrHFA-3	A10	BrRsCL3699s–KBrB06I03s	4.69	0.72	0.12	20

[a] Additive effect of the 'Shogoin-kabu' alleles on the number of hybrids per pollinated flower

[b] Dominance effect

[c] Phenotypic variance explained

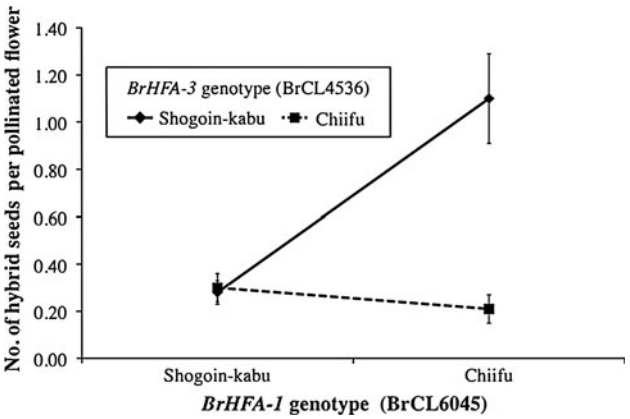

Fig. 4 The numbers of hybrid seeds per pollinated flower in four genotype classes of *qBrHFA-1* (BrCL4536) and *qBrHFA-3* (BrCL6045) in F_2 population derived from a *cross* between 'Shogoin-kabu' and 'Chiifu'

Therefore, to reveal epistatic interaction between these QTLs, the average hybrid formation abilities of different genotype classes in the F_2 populations were compared. For *qBrHFA-1* and *qBrHFA-3,* digenic interaction was detected by two-way ANOVA ($P < 0.0001$) (Fig. 4). A 'Chiifu' allele of BrCL6045 in *qBrHFA-1* was observed to increase the hybrid formation rate in 'Shogoin-kabu'-allele homozygotes and heterozygotes of BrCL4536 in *qBrHFA-3*, but not in 'Chiifu'-allele homozygotes of BrCL4536 (Supplementary Table 2). Moreover, the 'Shogoin-kabu'-allele of BrCL4536 in *qBrHFA-3* increased hybrid formation rates in heterozygotes of BrCL6045 in *qBrHFA-1*. These results suggest the existence of a synergistic epistatic effect between *qBrHFA-1* and *qBrHFA-3*.

Analysis of candidates for the genes controlling hybrid formation ability

The QTL regions of *qBrHFA-1* and *qBrHFA-3* were found to contain genes, *Bra023933* and *Bra002667,* homologous to the *A. thaliana* genes that have been identified to be involved in embryogenesis (Köhler et al. 2003). Nucleotide sequences of these genes of the 'Shogoin-kabu' and 'Chiifu' lines were determined. Many nucleotide polymorphisms between the 'Shogoin-kabu' and 'Chiifu' lines were detected in the coding regions of *Bra023933* located in *qBrHFA-1* region, which is a gene homologous to *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) (*At3g20740*), and of *Bra002667* located in *qBrHFA-3*, which is a gene homologous to *MULTICOPY SUPPRESSOR OF IRA 1* (*MSI1*) (*At5g58230*). In the 'Shogoin-kabu' allele of *Bra023933,* a single base insertion of adenine causing a frame shift was detected between 929 and 930 from the translation initiation site in the 11th exon (Fig. 5). In *Bra002667,* two single base insertions of adenine and thymine causing a frame shift were detected between 11 and 12 and between 17 and 18, respectively, from the translation initiation site in the first exon of the 'Chiifu' allele (Fig. 6). This sequence of the 'Chiifu' allele did not correspond to the *Bra002667* sequence of the published genome sequence of 'Chiifu' (Wang et al. 2011), but the 'Shogoin-kabu' sequence was the same as the published *Bra002667* sequence. This difference of the 'Chiifu' sequences might be due to variation in the cultivar 'Chiifu'. *Bra023933* and *Bra002667* were constantly expressed during ovule development after pollination and amounts of transcripts of *Bra023933* and *Bra002667* in ovules did not differ between the 'Shogoin-kabu' and 'Chiifu' lines.

Discussion

By intergeneric crossings of *B. rapa* pistils with *R. sativus* pollen, embryo abortion occurred and small-shriveled seeds with no germination ability were obtained. In the development of the small-shriveled seeds, shrinkage of embryo sacs was observed and embryos and endosperms did not develop. Although failure of fertilization as the cause of the

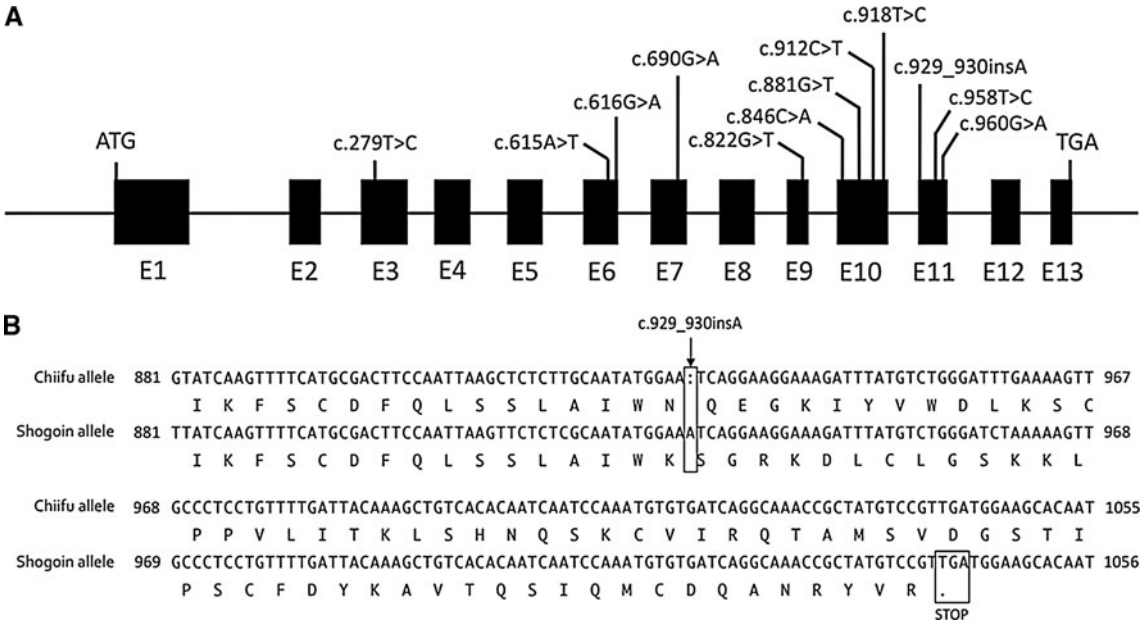

Fig. 5 Structure (**a**) and allele sequences (**b**) of gene *Bra023933*. SNPs and indels are shown with the nucleotide positions from the translation initiation site and bases of 'Chiifu' to the *left* and those of 'Shogoin-kabu' to the *right* of ">" above the black boxes indicating exons

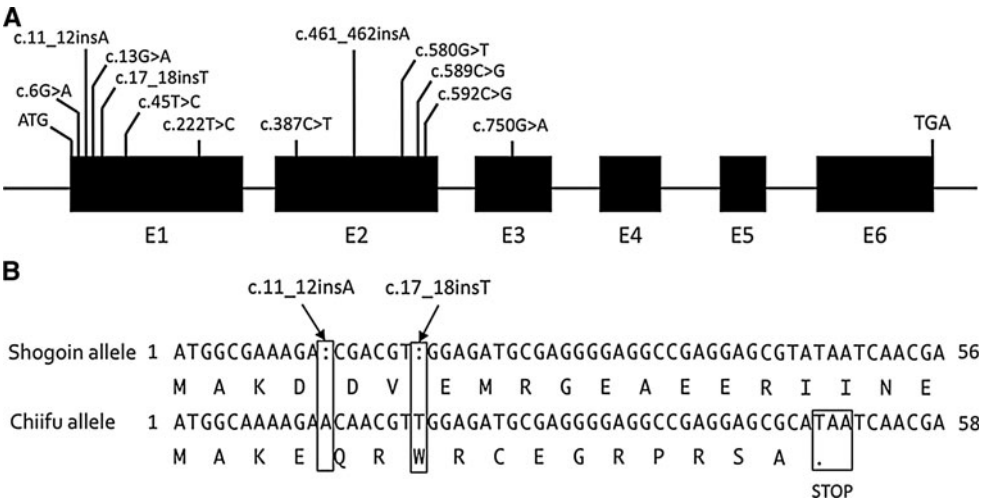

Fig. 6 Structure (**a**) and allele sequences (**b**) of gene *Bra002667*. SNPs and indels are shown with the nucleotide positions from the translation initiation site and bases of 'Shogoin-kabu' to the *left* and those of 'Chiifu' to the *right* of ">" above the black boxes indicating exons

unsuccessful development of embryos and endosperms cannot be ruled out, enlargement of siliques and ovules and the development of seed coats may suggest that the unsuccessful development of seeds is due to an arrest of embryo development at an early stage after fertilization. The early arrest of embryo development with shrunken embryo sacs has been reported (Bushell et al. 2003; Stoute et al. 2012), but development of small-shriveled seeds observed in the present study has not been previously reported.

In a 'Shogoin-kabu' line, a few small plump intergeneric hybrid seeds having germination ability were obtained together with the small-shriveled seeds. In the development of 'Shogoin-kabu' ovaries, two types of ovaries were found. In first type, embryo development was arrested at the heart stage, while enlargement of embryo sacs was observed. Although nuclear division occurred in endosperms, endosperm cellularization did not occur. Embryo development ceased and finally small-shriveled seeds with no germination ability were formed. A similar pattern of ovule development has been observed in interspecific crossings between *Arabidopsis thaliana* and *Arabidopsis arenosa* (Bushell et al. 2003) and interploidy crossings between hexaploids and diploids in *A. thaliana* (Scott et al. 1998). In the second type, shriveled ovaries and shrunken embryo sacs were observed, and embryo and endosperm developments were retarded, but were finally completed to maturity. Such a type of embryo development pattern has been observed in interploidy crossings in *A. thaliana* and in

B. oleracea (Scott et al. 1998; Stoute et al. 2012). Regarding these differences of seed sizes and shapes in interspecific or interploidy crossings, endosperm development after fertilization plays a major role, and genome imprinting in endosperms may be responsible for such differences (Scott et al. 1998; Berger and Chaudhury 2009). Similar differences of seed developments have been observed in mutants of imprinting genes (Köhler et al. 2003; Guitton et al. 2004). In the difference of hybrid seed formation ability of *B. rapa* in the intergeneric crossing with *R. sativus*, there might be a possibility of participation of genome imprinting.

Two QTLs for hybrid formation ability in the intergeneric crossings, i.e., *qBrHFA-1* and *qBrHFA-3*, were detected reproducibly in different populations grown in different years. *qBrHFA-1* showed the highest LOD score in 2008, and 'Shogoin-kabu' allele in this QTL had a negative effect on hybrid formation ability. On the other hand, *qBrHFA-3* had the highest LOD score in 2010, and 'Shogoin-kabu' allele in this QTL had a positive effect. The contrary effects of these QTLs suggest that the alleles of the 'Shogoin-kabu' lines have both positive and negative effects on hybrid formation ability. We found a synergistic epistatic effect between *qBrHFA-1* and *qBrHFA-3*. F_1 plants between the 'Shogoin-kabu' and 'Chiifu' lines and some F_2 plants derived from them formed 5 to 20 times as many hybrid seeds as the 'Shogoin-kabu' line, which is the parent having a high hybrid formation ability. This phenomenon can be explained as being due to the synergistic epistatic effect between *qBrHFA-1* and *qBrHFA-3*.

Evidence of epigenetic reprogramming during sexual reproduction has been reported (Gehring et al. 2009; Hsieh et al. 2009). Reproductive isolation is considered to be caused by differences between paternal and maternal epigenetic state (Jullien and Berger 2010). *Bra023933* and *Bra002667* located in the *qBrHFA-1* and *qBrHFA-3* regions were found to be homologous to *FIE* and *MSI1* in *A. thaliana*, respectively. *FIE* and *MSI1* encode proteins containing the WD40 domain involved in protein–protein interactions, and these proteins form a complex with various proteins to function in histone modification and interaction with chromatins at various developmental phases (Holec and Berger 2012). *MSI1* also encodes the *CAF1* domain, which is a component of a complex involved in chromatin assembly (Köhler et al. 2003; Hennig et al. 2005). In the endosperm development phase, FIE and MSI1 form the Polycomb Repressive Complex 2 (PRC2) with proteins encoded by maternal imprinting genes *MEDEA* (*MEA*) and *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*) regulated by an epigenetic mechanism, and control endosperm development by regulating expressions of paternal imprinting genes *PHERES 1 (PHE1)* and *AGAMOUS like 62 (AGL62)* by modification of histone methylation (Huh et al. 2008; Kang et al. 2008). Regulation of *PHE1* and *AGL62* by PRC2 is dosage dependent and influenced by ploidy level, gene expression levels of genes of PRC2 components, and species variation of the target site (Jossefson et al. 2006; Kinoshita 2007). In interploidy and interspecific crossings, hybrid seed formation is inferred to be prevented as a result of abnormality of endosperm development by mis-expression of the target genes due to imbalance between the number of their target genes and the amount of PRC2 complex (Jossefson et al. 2006; Kinoshita 2007). The 'Shogoin-kabu' allele of *qBrHFA-1* and the 'Chiifu' allele of *qBrHFA-2* had negative effects on hybrid formation ability, and insertions were found in exons. Because of frameshift mutations caused by these insertions, the 'Shogoin-kabu'-allele of *Bra023933* and the 'Chiifu'-allele of *Bra002667* were considered to be loss-of function alleles encoding truncated proteins. In addition, we showed the epistasis between *qBrHFA-1* and *qBrHFA-3*, which is similar to the interaction between *FIE* and *MSI1* in *A. thaliana* (Köhler et al. 2003). These results suggest that *Bra023933* and *Bra002667* homologous to *FIE* and *MSI1* might be involved in the hybrid formation ability of *B. rapa* lines in intergeneric crossings between *B. rapa* and *R. sativus*.

In this study, two QTLs for intergeneric hybrid formation ability of *B. rapa* and a combination of genotypes increasing this ability were identified, and genomic imprinting genes were suggested to be possible candidate genes responsible for the difference of the hybrid formation ability between two lines of *B. rapa*. Since DNA markers in the two QTLs were obtained, they can be used as markers for developing lines useful in introducing elite traits from wild species without embryo rescue techniques. For identification of the genes responsible for hybrid formation ability in the QTL regions, narrowing down the QTL region by developing near-isogenic lines and functional tests of *Bra023933* and *Bra002667* will be required.

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