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Research Paper

Characterization of cytoplasmic female sterility in an alloplasmic and monosomic addition line of *Brassica rapa* carrying the cytoplasm and one chromosome of *Diplotaxis tenuifolia*

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Alloplasmic plants exhibit various phenotypic changes such as cytoplasmic male sterility (CMS). We have been attempting to produce an alloplasmic *Brassica rapa* CMS line (2n = 20) carrying *Diplotaxis tenuifolia* cytoplasm (cyt-*Dt*) for several years, but a single extra chromosome always remained in all lines produced. We confirmed a *D. tenuifolia*-specific band in the alloplasmic line carrying *D. tenuifolia* cytoplasm by RAPD analysis, indicating that the additional chromosome was derived from *D. tenuifolia*. Here, we observed the phenotypic characteristics of the alloplasmic *B. rapa* monosomic addition line, named (cyt-*Dt*) *B. rapa* MAL, and investigated why a single extra chromosome is required in its genetic background for viability. When the (cyt-*Dt*) *B. rapa* MALs were crossed with pollen of several *B. rapa* lines, approximately 50% of the ovules attracted pollen tubes, and all the progeny had the additional chromosome. These results suggested that only the female gametes with n = 11 rather than n = 10 were fertilized and developed into mature seeds, and that cytoplasmic female sterility was overcome by nuclear restorer gene(s) derived from the cytoplasmic donor species.

Key Words: *Brassica rapa*, cytoplasmic female sterility, cytoplasmic male sterility, *Diplotaxis tenuifolia*, embryogenesis, intergeneric hybridization, pollen tube attraction.

Introduction

Many alloplasmic lines have been produced using methods such as intergeneric hybridization and somatic hybridization (Liu *et al.* 2005, Mwangangi *et al.* 2019, Yamagishi and Bhat 2014). Intergeneric hybridization with wild relatives is one of the most popular ways to produce alloplasmic lines in many crop plants. Substitution of organellar genomes induces various phenotypic changes such as cytoplasmic male sterility (CMS, Carlsson *et al.* 2008, Chase 2007), petaloid (Matsuzawa *et al.* 1999), pistillody (Murai *et al.* 2002), biomass improvement (Allen 2005, Soltani *et al.* 2016), germination capacity (Moison *et al.* 2010) and seed fecundity (Roux *et al.* 2016). The CMS phenotype has been utilized for producing hybrid seed in many crops (Chen and Liu 2014), while most of the other phenotypic changes have not been put to practical use.

Alien organellar genomes have been introduced into *Brassica rapa* (L.) (2n = 20, AA), mainly by intergeneric hybridization (Yamagishi and Bhat 2014). In the alloplasmic *B. rapa* lines low seed fertility, growth retardation and chlorosis were reported, as well as the CMS phenotype (Hinata and Konno 1979, Matsuzawa *et al.* 1999, Prakash and Chopra 1988). Thus, substitution of organellar genomes affects various developmental stages from vegetative growth to reproductive growth in *B. rapa*.

Diplotaxis tenuifolia (L.) (2n = 22, DtDt) is a wild Brassicaceae relative, also known as wild or sand rocket (Pignone 1997). When this organellar genome was introduced into *Raphanus sativus*, the CMS phenotype was not observed (Bang *et al.* 2003). We have been attempting to produce a *B. rapa* CMS line carrying *D. tenuifolia* cytoplasm (cyt-*Dt*) by intergeneric hybridization and successive backcrossings for several years, however, one of the *D. tenuifolia* chromosomes remained in all the lines produced. Similarly, a *Moricandia arvensis* chromosome was required for the production of an alloplasmic *B. rapa* CMS

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line carrying *M. arvensis* cytoplasm (cyt-*Ma*, Tsutsui *et al.* 2011). The (cyt-*Ma*) *B. rapa* (2n = 20) line degenerated before foliation because of severe chlorosis, while the (cyt-*Ma*) *B. rapa* (2n = 21) monosomic addition line showed a stable CMS phenotype without any abnormalities in vegetative growth or female fertility. Chlorosis was probably restored by the gene(s) located on the additional chromosome derived from *M. arvensis*.

Cross-talk between nuclear and organellar genomes greatly influences the development of alloplasmic lines. In this study, we investigated why the alloplasmic *B. rapa* MAL carrying *D. tenuifolia* cytoplasm requires the presence of one *D. tenuifolia* chromosome for viability.

Materials and Methods

Plant materials

The seed parent used was *D. tenuifolia* strain 1 (2n = 22, DtDt) that was provided by Cruciferae Genetic Stocks in Laboratory of Plant Breeding, Tohoku University, Japan. The 12 cultivars of *B. rapa* (2n = 20, AA) were used as the pollen parents; i.e. ssp. *perviridis* cv. 'Saori', cv. 'Hitomi', cv. 'Shousai', ssp. *chinensis* cv. 'Yohtei', cv. 'Ryokuyou', cv. 'Kazue', ssp. *pekinensis* cv. 'Kiraku 70', cv. 'Daifuku', cv. 'Harutourai' and ssp. *parachinensis* cv. 'Manamina'. They were provided by Tohoku Seed Company, Japan. *B. rapa* ssp. *pekinensis* cv. 'U-CC', ssp. *perviridis* cv. 'U-JMS' were accession lines in Laboratory of Plant Breeding, Utsunomiya University, Japan.

Production of hybrid progenies and amphidiploid plant

Interspecific hybridization between *D. tenuifolia* and *B. rapa* was performed using a bud pollination followed by embryo rescue. Flower buds were emasculated one day before flowering, immediately pollinated with fresh pollen and then bagged for approximately one week. Ovary culture followed by embryo culture was conducted for embryo rescue, according to Bang *et al.* (1996). In a step to produce amphidiploid plants, apical meristem tissue at plants extending major leaves has been wrapped with cotton soaked with 0.1% (w/v) colchicine solution for 48 hours. After this treatment, the other axillary buds except for treated apical meristem were removed for growing period. In development of the (cyt-*Dt*) BC_1 plants, we released bumblebees in a greenhouse placed *B. rapa* 9 cultivars with flowers growing, called open pollination method, after the bud pollinations. In successive backcrossings for development of the (cyt-*Dt*) BC_2—the (cyt-*Dt*) BC_8 plants, we used the bud pollination.

Identification of additional single chromosome

Total DNA of plants was extracted according to the CTAB method (Doyle and Doyle 1987). For a confirmation of the additional chromosome, we employed random amplified polymorphic DNA (RAPD) analysis using 400 RAPD-specific markers detectable random primers of 12-mer sequences (Common A to D, BEX, Tokyo, Japan) Amplification conditions were set according to Akaba *et al.* (2009). As a positive control marker of *B. rapa* genome, primer pair specific for *B. rapa* A1 (forward primer; 5′ GTGTTTCTCTTCAACGCCTTTT 3′, reverse primer; 5′ CACAAAGAATCCCCACAGATTT 3′, Cai *et al.* 2012) was used. The PCR products by the RAPD analysis were cloned into pGEM-T Easy Vector (Promega, Madison, USA) and were sequenced by an outsourced commission (Eurofins Genomics, Tokyo, Japan). Sequence reads were assembled and analyzed using CLC Sequence Viewer 7 (CLC Bio Qiagen, Aarhus, Denmark).

Cytogenetic investigation in hybrid progenies

Root tips of plants extending major leaves were preserved in 8-hydroxyquinolin solution during five hours, and then were fixed in Farmer solution (ethanol:acetic acid = 3:1) at 4°C overnight. Somatic chromosome number in the root tip cells from the (cyt-*Dt*) BC_1 plants to the (cyt-*Dt*) BC_8 plants was examined using Feulgen stain squash method followed by 1.0% (w/v) acetocarmine staining under an optical microscope (BX53LED, Olympus, Tokyo, Japan).

Meiotic chromosome behavior in pollen mother cells of the amphidiploid plants and the (cyt-*Dt*) BC_8 plants was observed using the 1.0% acetocarmine smear method under the optical microscope.

Pollen fertility was used as main criteria for the evaluation of fertile and sterile plants. The opened anthers were harvested, and the pollen grains were stained with 1.0% acetocarmine. The pollen fertility was ascertained by observing 1000 pollen grains under the optical microscope.

Anatomical analysis

To examine pattern of pollen tube growth, pistils at 48 hours after pollination were harvested and were fixed with the Farmer solution at 4°C overnight. They were rinsed with Milli-Q purified water and were softened with 1 N NaOH for about 30 minutes at 60°C. The samples were directly stained with 0.1% (w/v) aniline blue solution for more than one hour, and were observed under a fluorescence microscope (BX51N-33-FL2, Olympus, Tokyo, Japan). In order to obtain the image of many ovules, we performed a process of joining the multiple images. The rate of ovules with attracted pollen tube means the frequency for the total number of attracted pollen tubes (n) in at least 15 siliques per cross combination.

To compare embryonic development, siliques were collected at 10, 20 and 30 days after pollination (DAP). The collected fresh siliques were fixed in the Farmer solution at 4°C overnight after 10 minutes vacuum-infiltration. The samples of 10 and 20 DAP were treated with a graded ethanol series (90%, 70%, 50% and 30%) (v/v) at each 20 minutes, and were cleared in a transparent solution (chloral hydrate:glycerin:milli-Q = 8:2:1). The transparent samples were observed under a differential interference contrast microscopy (DIC) (BX53LED, Olympus, Tokyo,

Japan). The samples of 30 DAP were dissected and were observed under a stereo microscope (Leica S6 E, Leica, Wetzlar, Germany). The embryo development stages were classified into five stages: globular stage, heart stage, torpedo stage, walking stick stage and mature stage, according to Wilmar and Hellendoorn (1968).

Seed setting per silique was calculated as dividing the number of mature seeds by the average number of ovules.

Results

Production of alloplasmic B. rapa lines carrying D. tenuifolia cytoplasm

We performed intergeneric hybridization between *D. tenuifolia* and *B. rapa* cv. 'Saori', 'Yohtei', 'U-CC' and 'Manamina' (Mana.) (**Fig. 1**, **Supplemental Table 1**). We performed a diploidization treatment on four F_1 hybrid plants and selected an amphidiploid plant (2n = 42) carrying *D. tenuifolia* cytoplasm (cyt-*Dt*). We obtained 25 (cyt-*Dt*) sequidiploid BC_1 plants (2n = 31) using the open pollination method with nine cultivars, 'Saori', 'Hitomi', 'Shousai', 'Yohtei', 'Ryokuyou', 'Kazue', 'Kiraku 70', 'Daifuku' and 'Harutourai' (Haru.). Two hundred and seventy-two (cyt-*Dt*) BC_2 plants were produced by backcrossing with four cultivars, 'Saori', 'Shousai', 'U-JMS' and 'Yohtei'. We obtained four (cyt-*Dt*) BC_3 plants that included plants with 21 chromosomes by backcrossing using five cultivars, 'Saori', 'Shousai', 'Yohtei', 'Kazue' and 'Haru.'. We generated the (cyt-*Dt*) BC_4 to the (cyt-*Dt*) BC_7 plants by successive backcrossing using five random cultivars, 'Saori', 'Shousai', 'Yohtei', 'Kazue' and 'Haru.'. We conducted backcrossing independently with five cultivars, 'Saori', 'Shousai', 'Kazue', 'Haru.' and 'Mana.'. We counted somatic chromosome numbers in 145 individuals from the (cyt-*Dt*) BC_4 to the (cyt-*Dt*) BC_8 plants, with all surveyed plants containing an additional chromosome (**Table 1**, **Fig. 3A**). In theory, euploid plants (2n = 20) are generated at a probability of approximately 50%, however, we did not find any in our study. The single extra chromosome was inherited for at least four generations, and was common among the five different cultivars (**Table 1**).

We observed chromosome behavior during meiosis using pollen mother cells (PMCs) of the (cyt-*Dt*) BC_8 plants with extra chromosome(s). We found that 10 bivalents (10_{II}) and

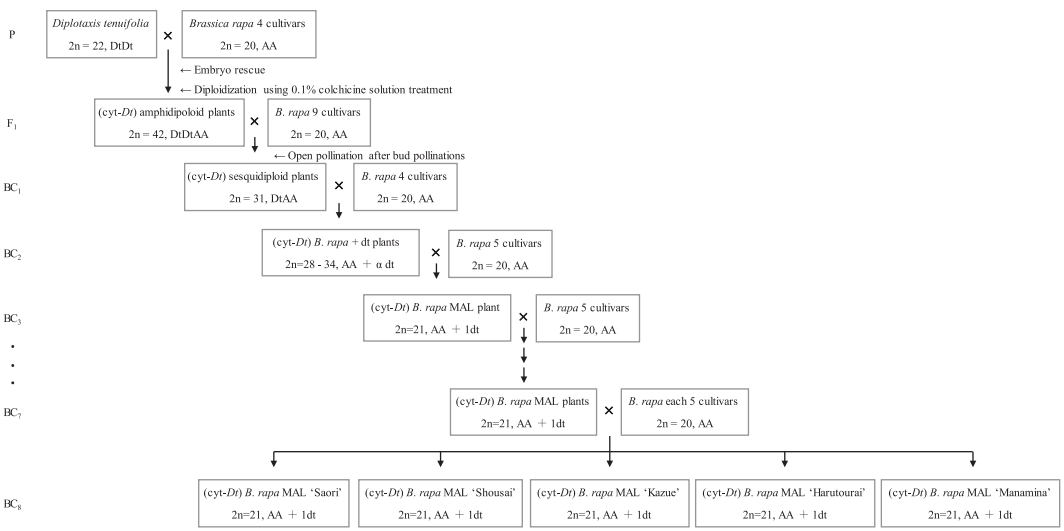

Fig. 1. Scheme for development to the (cyt-*Dt*) *B. rapa* MAL plant. The (cyt-*Dt*) *B. rapa* MAL plants were produced by intergenetic hybridization between *D. tenuifolia* and *B. rapa*, followed by successive backcrosses to *B. rapa*. The (cyt-*Dt*) means *D. tenuifolia* cytoplasm.

Table 1. Distribution of chromosome numbers of the (cyt-*Dt*) *B. rapa* MAL plants in BC_5 to BC_7 generations after successive backcrossings by multiple pollen parents of the *B. rapa* five cultivars, and in BC_8 generation after backcrossing by the individual *B. rapa* five cultivars

Generations	Cross combinations		Number of plants observed	Chromosome numbers (2n)	
	Seed parents (2n)	Pollen parents		20	21
BC_5	(cyt-*Dt*) *B. rapa* BC_4 plants (21)	*B. rapa* five cultivars	22	0	22
BC_6	(cyt-*Dt*) *B. rapa* BC_5 plants (21)	*B. rapa* five cultivars	15	0	15
BC_7	(cyt-*Dt*) *B. rapa* BC_6 plants (21)	*B. rapa* five cultivars	16	0	16
BC_8	(cyt-*Dt*) *B. rapa* BC_7 plants (21)	*B. rapa* 'Saori'	5	0	5
		B. rapa 'Shousai'	10	0	10
		B. rapa 'Kazue'	8	0	8
		B. rapa 'Harutourai'	60	0	60
		B. rapa 'Manamina'	9	0	9

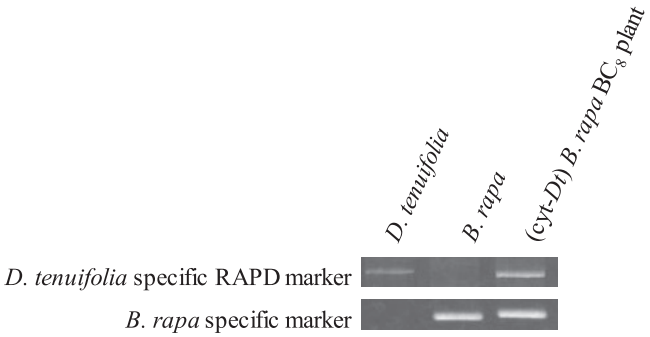

Fig. 2. PCR analysis using RAPD primers specific for *D. tenuifolia*. *B. rapa* specific marker is a positive control marker of *B. rapa* genome.

one univalent (1_I) were formed during metaphase I. A different number of chromosomes, 10 and 11, were formed at metaphase II (**Fig. 3B**, **3C**). This suggested that the 10 bivalents were derived from *B. rapa* genomes and that the one univalent was extra. The PMCs of the (cyt-*Dt*) BC_8 plants appeared to undergo meiosis regularly, and formed a microspore tetrad (**Fig. 3D**), with the result that half of the divided male gametes contained one extra chromosome, and the other half did not. These results suggest that this was the same for the female gametes.

To confirm the origin of the extra chromosome of the (cyt-*Dt*) BC_8 plants, we performed RAPD analysis using a number of 12-mer random primer sets. One of the primer sets amplified products of the same size in both *D. tenuifolia* and the (cyt-*Dt*) BC_8 plant (**Fig. 2**). These PCR products were subcloned and sequenced. Then, we searched for the most homologous *B.rapa* sequences in the database and compared them (**Supplemental Fig. 1**). We found 17 changes among the *B. rapa*, the *D. tenuifolia* and the (cyt-*Dt*) BC_8 plant sequences, consisting of 15 substitutions and two indels. All changes were common between the *D. tenuifolia* and the (cyt-*Dt*) BC_8 plant sequences, and different from the *B. rapa* sequence. Since the (cyt-*Dt*) BC_8 plants have been subjected to a considerable number of backcrossings with *B. rapa*, it is hard to imagine that this DNA fragment had undergone recombination with the chromosomes derived from *B. rapa*. The most likely explanation is that the (cyt-*Dt*) BC_8 plants had a full or partial chromosome derived from *D. tenuifolia*. Therefore, we named the (cyt-*Dt*) BC_8 plants with one chromosome of *D. tenuifolia*, the (cyt-*Dt*) *B. rapa* monosomic addition line (MAL).

Flower organ morphology and fertility of the (cyt-Dt) B. rapa MAL

We investigated the phenotypes of the (cyt-*Dt*) *B. rapa* MAL, focusing on flower morphology and fertility. The flowers of the (cyt-*Dt*) *B. rapa* MAL were slightly smaller than those of the euplasmic *B. rapa* line (**Fig. 3E–3H**). The flowers of the (cyt-*Dt*) *B. rapa* MAL had shrunken stamens containing short filaments (**Fig. 3H**). The anthers of the euplasmic *B. rapa* line contained abundant pollen grains,

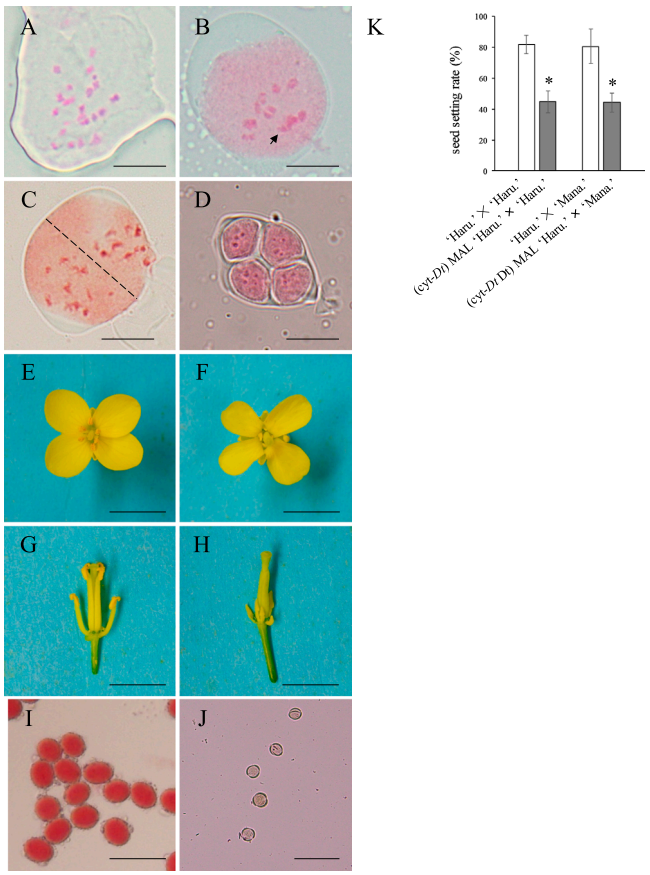

Fig. 3. Cytogenetical and morphological characteristics of the (cyt-*Dt*) *B. rapa* MAL plant. (A) Somatic chromosome numbers of the root tip of the (cyt-*Dt*) *B. rapa* MAL 'Haru.' ($2n = 21$). (B–D) Chromosome behavior during meiosis of the (cyt-*Dt*) *B. rapa* MAL 'Haru.' in metaphase I ($10_{II} + 1_I$) (B), metaphase II ($10 + 11$) (C) and pollen tetrad stage (D). Arrow indicates one univalent in (B). (E–H) Floral morphology of the euplasmic *B. rapa* 'Haru.' (left) and the (cyt-*Dt*) *B. rapa* MAL 'Haru.' (right). (I) Fertile pollen grains of the euplasmic *B. rapa* 'Haru.'. (J) Sterile pollen grains of the (cyt-*Dt*) *B. rapa* MAL 'Haru.'. Scale bars: 10 μm in (A–D), 5.0 mm in (E–H), 50 μm in (I, J). (K) Seed setting rates in the euplasmic *B. rapa* 'Haru.' (white) and the (cyt-*Dt*) *B. rapa* 'Haru.'(grey). The data show as means ± SD, $n = 10$. Significant differences ($p < 0.001$) between the euplasmic *B. rapa* line and the (cyt-*Dt*) *B. rapa* MAL are indicated by *.

which were strongly stained by acetocarmine (**Fig. 3I**). In contrast to this, the anthers of the (cyt-*Dt*) *B. rapa* MAL contained few pollen grains, which were not stained and were not scattered at all from the anthers during the flowering period (**Fig. 3J**). Therefore, all male gametophytes from the (cyt-*Dt*) *B. rapa* MAL lost their fertility, and the (cyt-*Dt*) *B. rapa* MAL showed a complete CMS phenotype. The other organs, including nectarines and pistils, looked no different from those of the euplasmic *B. rapa* line. The seed setting rate of the (cyt-*Dt*) *B. rapa* MAL 'Haru.' was approximately half of that of the euplasmic *B. rapa* 'Haru.' (**Fig. 3K**). To clarify whether the decline in the seed setting rate of the (cyt-*Dt*) *B. rapa* MAL is due to self-incompatibility (SI), ovules of the (cyt-*Dt*) *B. rapa* MAL

were fertilized with pollen from another subspecies (**Fig. 3K**, **Supplemental Fig. 2A**). The seed setting rate was approximately 50% that obtained after crossing within the same species, confirming that the influence of SI was small.

The ability to attract pollen tube in the (cyt-Dt) B. rapa MAL

To further clarify why seed setting has declined in the (cyt-*Dt*) *B. rapa* MAL, we observed the ability of the (cyt-*Dt*) *B. rapa* MAL to attract pollen tubes in pistils (**Fig. 4**). Almost all ovules of the euplasmic *B. rapa* 'Haru.' attracted pollen tube (**Fig. 4A**, **4C**), while approximately half of ovules failed to attract pollen tube in the (cyt-*Dt*) *B. rapa* MAL 'Haru.' (**Fig. 4B**, **4C**). Similar data was acquired when crossing with other subspecies (**Fig. 4C**, **Supplemental Fig. 2B**), indicating that the decline was not influenced by SI. The decline in seed setting in the (cyt-*Dt*) *B. rapa* MAL is most likely associated with the fact that half the ovules lack the ability to attract pollen tube, indicating female sterility.

Embryo development in the (cyt-Dt) B. rapa MAL

We investigated the development of fertilized ovules in the (cyt-*Dt*) *B. rapa* MAL 'Haru.' (**Fig. 5**). The frequency of ovule enlargement in the euplasmic *B. rapa* 'Haru.' remained stable at 10, 20 and 30 DAP (**Fig. 5A**), and that of the (cyt-*Dt*) *B. rapa* MAL 'Haru.' also remained stable, at a level half that of the euplasmic *B. rapa* 'Haru.'. There was no difference in the size of the enlarged ovules between the (cyt-*Dt*) *B. rapa* MAL and the euplasmic *B. rapa* line at all time points tested. Most of the fertilized ovules kept growing for at least 30 DAP in both lines. We classified the embryonic stage of each fertilized ovule into five classes in both lines at three different time points (**Fig. 5H**, **5I**). Although embryogenesis of the (cyt-*Dt*) *B. rapa* MAL 'Haru.' was slightly delayed compared to the euplasmic *B. rapa* 'Haru.' at 20 DAP (**Fig. 5H**, **5I**), the rate of formation of mature embryos in the (cyt-*Dt*) *B. rapa* MAL 'Haru.' at 30 DAP was similar to that of the euplasmic *B. rapa* 'Haru.'. This result also suggested that almost all fertilized ovules can also survive and keep developing in the (cyt-*Dt*) *B. rapa* MAL. Thus, the fertilized ovules in the (cyt-*Dt*) *B. rapa* MAL 'Haru.', accounting for approximately 50% of the total ovules, were able to develop into mature seeds. In short, the cause of the decrease in the seed setting level in the (cyt-*Dt*) *B. rapa* MAL can be mostly explained by the fact that almost half the ovules do not attract pollen tube. Therefore, pre-zygotic defects rather than post-zygotic defects are involved in female fertility and progeny development of the (cyt-*Dt*) *B. rapa* MAL.

Although female gametes in the (cyt-*Dt*) *B. rapa* MAL theoretically segregate into two chromosome numbers, n = 10 and n = 11, at the same rate, all the progeny always have an additional chromosome derived from *D. tenuifolia*, resulting in 2n = 21. This implies that female fertility was probably restored by the gene(s) located on the additional chromosome derived from *D. tenuifolia*. We suggest a possible model in which only the female gametes with an additional chromosome are able to survive (**Fig. 6**).

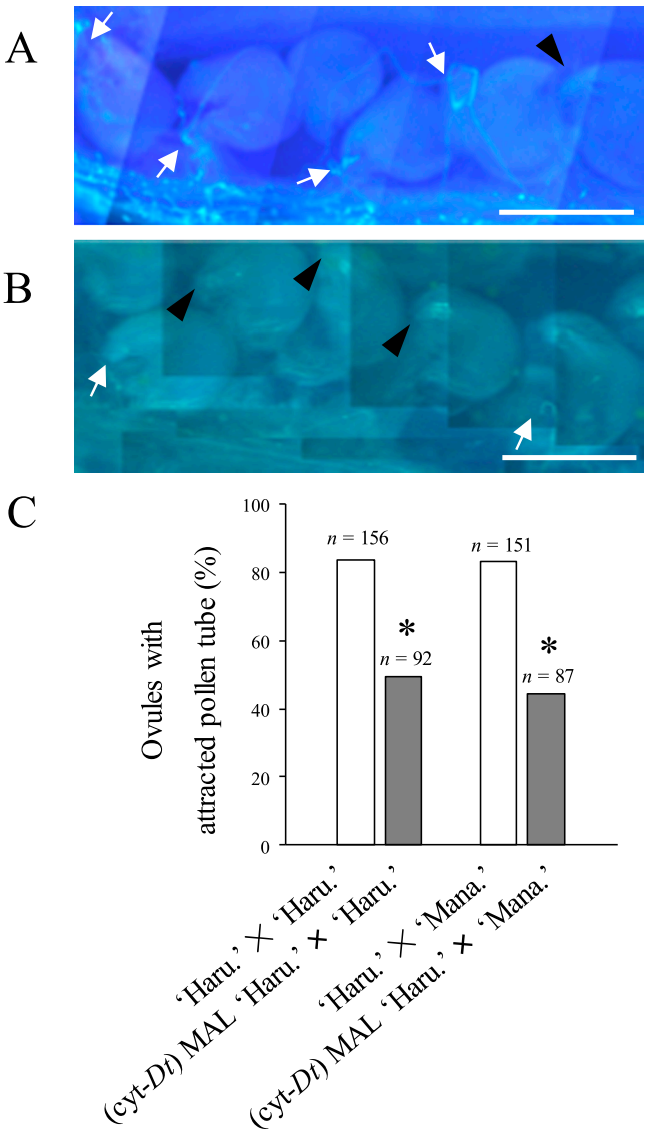

Fig. 4. The pollen tubes attraction ability of the (cyt-*Dt*) *B. rapa* MAL. (A) Pollen tubes in sib pollination of the euplasmic *B. rapa* 'Haru.'. (B) Pollen tubes in the (cyt-*Dt*) *B. rapa* MAL 'Haru.' × the euplasmic *B. rapa* 'Haru.'. Arrows indicate the ovules with attracted pollen tube. Arrowheads indicate the ovules without attracted pollen tube. Scale bars: 50 µm. (C) Rates of ovules with attracted pollen tube. Significant differences ($p < 0.001$) between the euplasmic *B. rapa* line and the (cyt-*Dt*) *B. rapa* MAL are indicated by *.

Discussion

In various crop plants, alloplasmic lines have been developed to obtain agriculturally useful traits such as CMS (Budar and Berthomé 2007). Alloplasmic lines are difficult to produce in some species combinations, however, due to

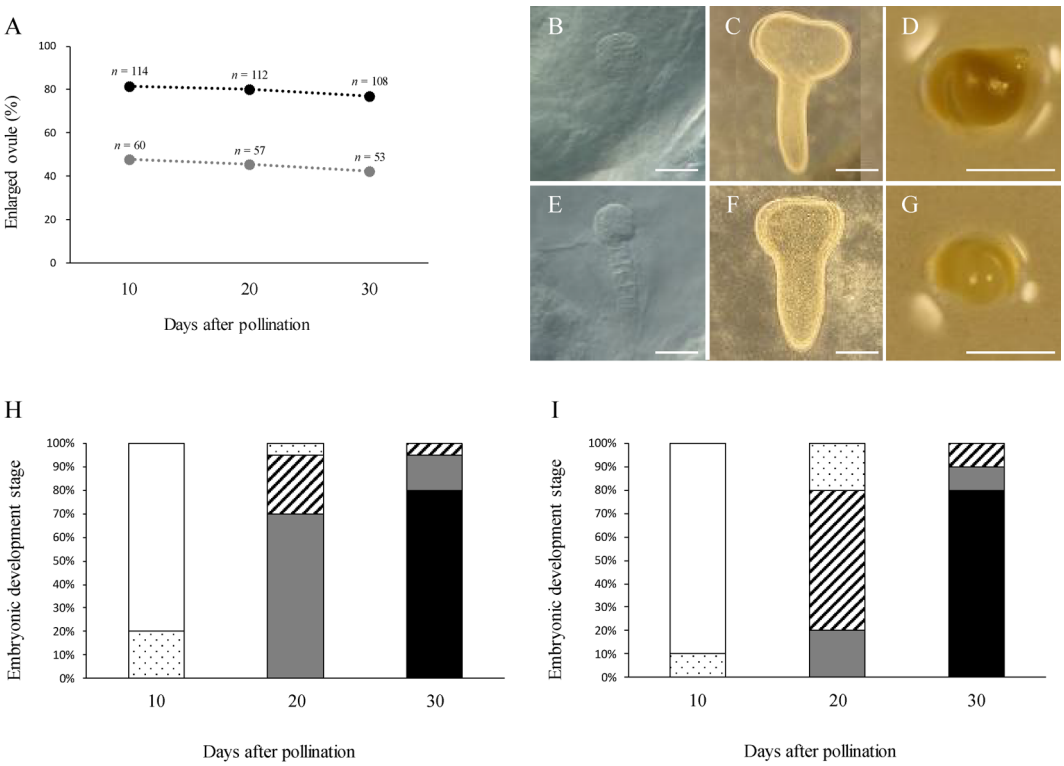

Fig. 5. The embryo development ability in the (cyt-*Dt*) *B. rapa* MAL plant. (A) Number of enlarged ovules of sib pollination of the euplasmic *B. rapa* 'Haru.' (black) and the (cyt-*Dt*) MAL 'Haru.' × the euplasmic *B. rapa* 'Haru.' (gray). The data are the frequencies for the total number of enlarged ovules (*n*) in at least 15 siliques per combination. (B–G) Embryo development of sib crossing of the (cyt-*Dt*) *B. rapa* MAL 'Haru.' (B–D) and the one of crossing combination of the (cyt-*Dt*) *B. rapa* MAL 'Haru.' × the euplasmic *B. rapa* 'Haru.' (E–G) at 10 days after pollination (B, E), 20 days after pollination (C, F) and 30 days after pollination (D, G). Scale bars: 100 µm in (B, E), 250 µm in (C, F), 500 µm in (D, G). (H) Embryonic development stage of sib crossing of the euplasmic *B. rapa* 'Haru.'. (I) The (cyt-*Dt*) *B. rapa* MAL 'Haru.' × the euplasmic *B. rapa* 'Haru.'. The embryonic development stage means five classified stages; globular stage (blanked square), heart stage (dotted square), torpedo stage (hatched square), walking stick stage (gray square), mature stage (black square). *n* = 20.

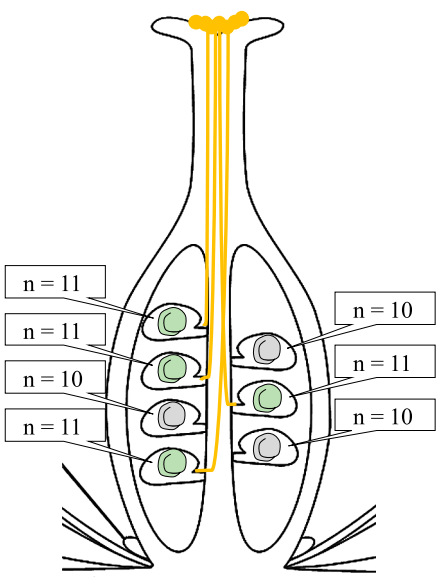

Fig. 6. A model for understanding seed formation in the (cyt-*Dt*) *B. rapa* MAL plant. Ovules having the additional chromosome (n = 11) can attract pollen tube, while the other ovules without additional chromosome (n = 10) cannot attract one.

incompatibilities between the organellar and nuclear genomes. The retention of a single chromosome derived from the cytoplasmic donor species may help in these cases. For example, in wheat, some alloplasmic lines, even after a large number of backcrosses, retain an additional chromosome originating from their cytoplasmic donor species (Endo and Tsunewaki 1975, Maan 1976, Tsujimoto *et al.* 1987). Retaining a chromosome derived from a cytoplasmic donor also allows progeny to be obtained from alloplasmic lines in Brassicaceae (Tsutsui *et al.* 2011). Our study suggested that an additional chromosome derived from *D. tenuifolia* was required in the alloplasmic *B. rapa* plants to restore cytoplasmic female sterility (CFS) during female gamete developmental stages. Since the (cyt-*Dt*) *B. rapa* MAL exhibited stable CMS regardless of the presence or absence of the additional chromosome, the additional chromosome was not involved in controlling the fertility of male gametes.

The ovules that became enlarged in the (cyt-*Dt*) *B. rapa* MAL developed into mature embryos at 30 DAP (**Fig. 5A**, **5I**). This suggests that embryogenesis occurred normally in the ovules, and that the additional chromosome may play a

role before fertilization. The ovules lacking the additional chromosome had a defect at some point during the female gametophyte stages, reducing the number of progeny by about half. Though our data clarified the inability of (cyt-*Dt*) *B. rapa* MAL to attract pollen tubes, the additional chromosome may contribute to female gametogenesis, as well as contributing directly to pollen tube attraction. The inability of some ovules to attract pollen tubes may be caused by immaturity of the female gametes. However, the type of defects occurring in the embryo sac of the (cyt-*Dt*) *B. rapa* MAL remain unknown. It is known that the many genes related to plant development are regulated by the coordination anterograde (nuclear to mitochondrial) and retrograde (mitochondrial to nuclear) signaling pathways (Ng *et al.* 2014). For example, many nuclear-encoded pentatricopeptide repeat (PPR) proteins involve in organellar gene expression in plants (Lurin *et al.* 2004), and several PPR genes are essential for embryonic development (Cushing *et al.* 2005). In addition to PPR proteins, several signal molecules originating from chloroplasts or mitochondria are known to modulate the expression of nuclear genes (Isemer *et al.* 2012, Koussevitzky *et al.* 2007, Sun *et al.* 2011). Indispensable signal molecules in the nuclear genome corresponding to the organellar genomes of *D. tenuifolia* may be located on the additional chromosome of the (cyt-*Dt*) *B. rapa* MAL. Further investigations will reveal how the alien organellar genome is regulated by the factor(s) encoded in the additional chromosome.

The (cyt-*Dt*) *B. rapa* MAL exhibited a CMS phenotype, and developed normally until the flowering stage without a chlorotic phenotype. Therefore, the (cyt-*Dt*) *B. rapa* MAL could be utilized for F_1 seed production in *B. rapa* as a new CMS system, once we introduce the restoration factor(s) responsible for female sterility on the additional chromosome into the *B. rapa* genome. This method has been previously reported, for example, the CMS phenotype was restored by introgression of nuclear restorer gene(s) derived from *M. arvensis* in *B. juncea* CMS plants carrying *M. arvensis* cytoplasm (Kirti *et al.* 1992, Prakash *et al.* 1998). Development of *B. oleracea* lines carrying *R. sativus* cytoplasm using successive backcrossing methods was interrupted due to its complete female sterility (McCollum 1979). The lines were finally developed by embryo rescue (Bannerot *et al.* 1974) and utilization of a tetraploid pollen parent (McCollum 1981). The (cyt-*Dt*) *B. rapa* CMS plants should be developed using a variety of approaches in order to reduce the risks associated with using a single CMS system.

In conclusion, we showed that only half of the female gametes of the (cyt-*Dt*) *B. rapa* MAL are female-fertile and these only survive due to the addition of a single chromosome. This study provides a new use for the cross-talk between the nuclear and organellar genomes in Brassicaceae.

Author Contribution Statement

Y. F., T. O. and S. W. B. conceived the project and designed the research. S. S. and S. W. B. developed the plants in this study. Y. F. conducted the experiments, analyzed the data and prepared first draft of the manuscript. Y. N. and M. Y. partly participated in the experiments. T. O. and S. W. B. critically reviewed and improved the final manuscript.

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Literature Cited

Akaba, M., Y. Kaneko, Y. Ito, Y. Nakata, S.W. Bang and Y. Matsuzawa (2009) Production and characterization of *Brassica napus–Raphanus sativus* monosomic addition lines mediated by the synthetic amphidiploid "*Raphanobrassica*". Breed. Sci. 59: 109–118.

Allen, J.O. (2005) Effect of teosinte cytoplasmic genomes on maize phenotype. Genetics 169: 863–880.

Bang, S.W., Y. Kaneko and Y. Matsuzawa (1996) Production of intergeneric hybrids between *Raphanus* and *Sinapis* and the cytogenetics of their progenies. Breed. Sci. 46: 45–51.

Bang, S.W., Y. Mizuno, Y. Kaneko, Y. Matsuzawa and K.S. Bang (2003) Production of intergeneric hybrids between the C_3-C_4 intermediate species *Diplotaxis tenuifolia* (L.) DC. and *Raphanus sativus* L. Breed. Sci. 53: 231–236.

Bannerot, H., L. Boulidard, Y. Cauderon and J. Tempe (1974) Transfer of cytoplasmic male sterility from *Raphanus sativus* to *Brassica oleracea*. Proc. Eucarpia Meeting Cruciferae: 52–54.

Budar, F. and R. Berthomé (2007) Cytoplasmic male sterilities and mitochondrial gene mutations in plants. Plant mitochondria: annual plant reviews 31: 278–307.

Cai, G., Q. Yang, Q. Yang, Z. Zhao, H. Chen, J. Wu, C. Fan and Y. Zhou (2012) Identification of candidate genes of QTLs for seed weight in *Brassica napus* through comparative mapping among *Arabidopsis* and *Brassica* species. BMC Genet. 13: 105.

Carlsson, J., M. Leino, J. Sohlberg, J.F. Sundstrom and K. Glimelius (2008) Mitochondrial regulation of flower development. Mitochondrion 8: 74–86.

Chase, C.D. (2007) Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. Trends Genet. 23: 81–90.

Chen, L. and Y.G. Liu (2014) Male sterility and fertility restoration in crops. Annu. Rev. Plant Biol. 65: 579–606.

Cushing, D.A., N.R. Forsthoefel, D.R. Gestaut and D.M. Vernon (2005) *Arabidopsis emb175* and other *ppr* knockout mutants reveal essential roles for pentatricopeptide repeat (PPR) proteins in plant embryogenesis. Planta 221: 424–436.

Doyle, J.J. and J.L. Doyle (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11–15.

Endo, T.R. and K. Tsunewaki (1975) Sterility of common wheat with

Aegilops triuncialis cytoplasm. J. Hered. 66: 13–18.

Hinata, K. and N. Konno (1979) Studies on a male sterile strain having the *Brassica campestris* nucleus and the *Diplotaxis muralis* cytoplasm. I On the breeding procedure and some characteristics of the male sterile strain. Japan. J. Breed. 29: 305–311.

Isemer, R., M. Mulisch, A. Schäfer, S. Kirchner, H.U. Koop and K. Krupinska (2012) Recombinant Whirly1 translocates from transplastomic chloroplasts to the nucleus. FEBS Lett. 586: 85–88.

Kirti, P., S. Narasimhulu, S. Prakash and V. Chopra (1992) Somatic hybridization between *Brassica juncea* and *Moricandia arvensis* by protoplast fusion. Plant Cell Rep. 11: 318–321.

Koussevitzky, S., A. Nott, T.C. Mockler, F. Hong, G. Sachetto-Martins, M. Surpin, J. Lim, R. Mittler and J. Chory (2007) Signals from chloroplasts converge to regulate nuclear gene expression. Science 316: 715–719.

Liu, J., X. Xu and X. Deng (2005) Intergeneric somatic hybridization and its application to crop genetic improvement. Plant Cell Tissue Organ Cult. 82: 19–44.

Lurin, C., C. Andres, S. Aubourg, M. Bellaoui, F. Bitton, C. Bruyere, M. Caboche, C. Debast, J. Gualberto, B. Hoffmann *et al.* (2004) Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell 16: 2089–2103.

Maan, S.S. (1976) Cytoplasmic homology between *Aegilops squarrosa* and *Ac. cylindrica* Host. Crop Sci. 16: 757–761.

Matsuzawa, Y., S. Mekiyanon, Y. Kaneko, S. Bang, K. Wakui and Y. Takahata (1999) Male sterility in alloplasmic *Brassica rapa* L. carrying *Eruca sativa* cytoplasm. Plant Breed. 118: 82–84.

McCollum, G.D. (1979) Sterility in successive backcrosses of *Raphanobrassica* (2n = 4x = 36) with recurrent *Brassica oleracea* (2n = 2x = 18). Can. J. Genet. Cytol. 21: 479–485.

McCollum, G.D. (1981) Induction of an alloplasmic male-sterile *Brassica oleracea* by substituting cytoplasm from 'Early Scarlet Globe' radish (*Raphanus sativus*). Euphytica 30: 855–859.

Moison, M., F. Roux, M. Quadrado, R. Duval, M. Ekovich, D.H. Le, M. Verzaux and F. Budar (2010) Cytoplasmic phylogeny and evidence of cyto-nuclear co-adaptation in *Arabidopsis thaliana*. Plant J. 63: 728–738.

Murai, K., S. Takumi, H. Koga and Y. Ogihara (2002) Pistillody, homeotic transformation of stamens into pistil-like structures, caused by nuclear–cytoplasm interaction in wheat. Plant J. 29: 169–181.

Mwangangi, I.M., J.K. Muli and J.O. Neondo (2019) Plant hybridization as an alternative technique in plant breeding improvement. Asian J. Res. Crop Sci. 4: 1–11.

Ng, S., I. De Clercq, O. Van Aken, S.R. Law, A. Ivanova, P. Willems, E. Giraud, F. Van Breusegem and J. Whelan (2014) Anterograde and retrograde regulation of nuclear genes encoding mitochondrial proteins during growth, development, and stress. Mol. Plant 7: 1075–1093.

Pignone, D. (1997) Present Status of Rocket Genetic Resources and Conservation Activities. *In*: Pignone, D. and S. Padulosi (eds.) Rocket a Mediterranean crop for the world. Report of a workshop 13–14. December 1996, Legnaro Italy, IPGRI, Rome, pp. 2–12.

Prakash, S. and M. Chopra (1988) Synthesis of alloplasmic *Brassica campestris* as a new source of cytoplasmic male sterility. Plant Breed. 101: 253–255.

Prakash, S., P. Kirti, S. Bhat, K. Gaikwad, V.D. Kumar and V. Chopra (1998) A *Moricandia arvensis*–based cytoplasmic male sterility and fertility restoration system in *Brassica juncea*. Theor. Appl. Genet. 97: 488–492.

Roux, F., T. Mary-Huard, E. Barillot, E. Wenes, L. Botran, S. Durand, R. Villoutreix, M.L. Martin-Magniette, C. Camilleri and F. Budar (2016) Cytonuclear interactions affect adaptive traits of the annual plant *Arabidopsis thaliana* in the field. Proc. Natl. Acad. Sci. USA 113: 3687–3692.

Soltani, A., A. Kumar, M. Mergoum, S.M. Pirseyedi, J.B. Hegstad, M. Mazaheri and S.F. Kianian (2016) Novel nuclear-cytoplasmic interaction in wheat (*Triticum aestivum*) induces vigorous plants. Funct. Integr. Genomics 16: 171–182.

Sun, X., P. Feng, X. Xu, H. Guo, J. Ma, W. Chi, R. Lin, C. Lu and L. Zhang (2011) A chloroplast envelope-bound PHD transcription factor mediates chloroplast signals to the nucleus. Nat. Commun. 2: 477.

Tsujimoto, H., I. Panayotov and K. Tsunewaki (1987) Behavior of an extra chromosome carried by alloplasmic common wheat lines having *Agropyron trichophorum* cytoplasm. Jap. J Genet. 62: 291–299.

Tsutsui, K., B.H. Jeong, Y. Ito, S.W. Bang and Y. Kaneko (2011) Production and characterization of an alloplasmic and monosomic addition line of *Brassica rapa* carrying the cytoplasm and one chromosome of *Moricandia arvensis*. Breed. Sci. 61: 373–379.

Wilmar, J. and M. Hellendoorn (1968) Embryo culture of Brussels sprouts for breeding. Euphytica 17: 28–37.

Yamagishi, H. and S.R. Bhat (2014) Cytoplasmic male sterility in Brassicaceae crops. Breed. Sci. 64: 38–47.