Production and characterization of *Brassica napus*–*Raphanus sativus* monosomic addition lines mediated by the synthetic amphidiploid “*Raphanobrassica*”

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In order to breed *Brassica napus*–*Raphanus sativus* monosomic addition lines (MALs), hybridizations between two synthetic amphidiploid (RRAA and RRCC) and *B. napus* (AACC) were performed. Two allo-octoploids (RRAAAACC and AACCRRC) were produced from each F₁ hybrid by chromosome doubling. From successive backcrosses to *B. napus*, MAL plants were first obtained in BC₂ generation and the *R. sativus*-derived chromosome was identified by genomic *in situ* hybridization (GISH). The nine chromosomes of R-genome in the MAL plants were clearly classified by each chromosome-specific RAPD markers. As a result, alloplasmic (radish cytoplasm) *B. napus*–*R. sativus* MAL having 8 types (a–i, except for h-type) and autoplasmic (rape cytoplasm) MAL with complete 9 types (a–i) were obtained in BC₃ and BC₄ generations. These alloplasmic and autoplasmic MAL plants were showed differences in their morphological, physiological and cytogenetical characters. From the survey of favorable traits, it was suggested that the a-type had fertility restoring gene(s) for male sterility in alloplasmic line and the g-type had a gene controlling white color petal. These two MALs are useful materials for exploring agronomic traits located on each chromosome of radish and for promoting the introgression of promising radish gene(s) to *B. napus*.

Key Words: *Raphanus sativus*, *Brassica napus*, *Raphanobrassica*, monosomic addition lines (MALs), random amplified polymorphic DNA (RAPD), genomic *in situ* hybridization (GISH).

Introduction

Although radish (*Raphanus sativus* L. 2n=18, RR genome) is one of the most important vegetables in East Asia (Kaneko *et al.* 2007), rape (*Brassica napus* L. 2n=38, AACC genome), which includes winter and spring oilseed, fodder and vegetables rape forms, is today the most widely cultivated crop species in the crucifer family (Brassicaceae) (Snowdon *et al.* 2007). Radish possesses agronomically useful traits such as resistances to beet cyst nematode (*Heterodera schachtii*) (Lelivelt and Krens 1992, Peterka *et al.* 2004), clubroot (*Plasmodiophora brassicae*) (Ashizawa *et al.* 1980) and white rust (*Albugo candida*) (Kolte *et al.* 1991) and cytoplasmic male sterility (CMS) (Ogura 1968, Ikegaya 1986a, 1986b). Other *Brassicaceae* crops often lack these traits, and a large number of rape cultivars are susceptible to beet cyst nematode (Peterka *et al.* 2004) and clubroot (Ashizawa *et al.* 1980). Therefore, radish can be used as a nuclear and/or cytoplasm donor plant for the modification of rape.

A number of intergeneric hybridizations between rape and radish have been performed in order to introduce agro-

nomically useful traits of radish to rape, but only a few hybrid plants were obtained due to their poor crossability (Dolstra 1982). To overcome these poor crossability, several breeding methods have been performed: ovary culture (Luo *et al.* 1989), ovule culture (Dolstra 1982), sequential culture (Lelivelt *et al.* 1993), flower-culture (Metz *et al.* 1995), *in vitro* organ culture (Luo *et al.* 2000), and protoplast fusion (Pelletier *et al.* 1983, Sakai and Imamura 1990, Lelivelt and Krens 1992, Sakai *et al.* 1996, Wang *et al.* 2006). Furthermore, it has been also difficult to produce backcross progenies in this cross-combination (Dolstra 1982). On the other hand, it is relatively easy to obtain F₁ hybrids and backcross progenies by the hybridization between *Brassicoraphanus* (*B. rapa* × *R. sativus*, 2n=38, AARR genome) and *B. napus*, when *Brassicoraphanus* was used as a bridging plant (Dolstra 1982, Paulmann and Röbbelen 1988).

The addition lines of a single alien chromosome to the full complement of the recipient species are called monosomic addition lines (MALs), and morphological and physiological features of MALs are affected by an alien chromosome (Khush 1973). Since O'Mara (1940) had first produced MAL in wheat, it has been successfully bred in many crops. In *Brassicaceae* with a number of important agricultural crops, several MAL series have been successfully bred through interspecific and intergeneric hybridizations to

Communicated by T. Terachi

Received August 25, 2008. Accepted February 19, 2009.

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express their phenotypic characteristics (Kaneko *et al.* 1987, 2001, Quiros *et al.* 1987, 1988, Jahier *et al.* 1989, Struss *et al.* 1991, Chen *et al.* 1992, Srinivasan *et al.* 1998, Bang *et al.* 2002, Peterka *et al.* 2004). These MALs have been also analyzed to locate the agronomic traits and gene(s) on the added chromosome (Zhu *et al.* 1993, Chèvre *et al.* 1996, 1997, Kaneko *et al.* 1996, Chen *et al.* 1997, Peterka *et al.* 2004, Bang *et al.* 2009). However, only a few studies report the production of alloplasmic and autoplasmic MALs with the same nuclear background (Budahn *et al.* 2008, Bang *et al.* 2009).

Recently, Budahn *et al.* (2008) reported the production of alloplasmic and autoplasmic *B. napus*–*R. sativus* MALs with radish and rape cytoplasms, respectively. However, no detailed genetic analyses have been performed to characterize each type of these two MALs.

In this study, we tried to breed alloplasmic and autoplasmic *B. napus*–*R. sativus* MALs ($2n=39$) by crossing between *B. napus* and two synthetic amphidiploids “*Raphanobrassica*”: RA-89 (*R. sativus* × *B. rapa*, $2n=38$, RRAA, Matsuzawa *et al.* 2000) and Rb-63 (*R. sativus* × *B. oleracea*, $2n=36$, RRCC, Matsuzawa *et al.* 1985), as the bridging plants to donate the radish chromosome and cytoplasm into *B. napus* ($2n=38$, AACC). Each *R. sativus* chromosome in MALs was identified by random amplified polymorphic DNA (RAPD) analysis and cytogenetical observations including genomic *in situ* hybridization (GISH). Morphological and physiological features of the added chromosomes were also studied to characterize and maintain these two MALs.

Materials and Methods

Plant materials

Two synthetic amphidiploids, *Raphanobrassica* ‘RA-89’ ($2n=38$, RRAA) and ‘Rb-63’ ($2n=36$, RRCC) are stocks of the Laboratory of Plant Breeding, Utsunomiya University, Japan. *Raphanobrassica* ‘RA-89’ was developed by hybridization of *R. sativus* L. cv. ‘Risou’ ($2n=18$, RR) and *B. rapa* L. ssp. *Japonica* cv. ‘Kyo-mizuna’ ($2n=20$, AA) (Matsuzawa *et al.* 2000). *Raphanobrassica* ‘Rb-63’ was developed from crossing between *R. sativus* L. cv. ‘Shogoin’ ($2n=18$, RR) and *B. oleracea* L. var. *acephala* cv. ‘Murasaki-habotan’ ($2n=18$, CC) (Matsuzawa *et al.* 1985). *Brassica napus* strain ‘N-350’ used for the recurrent parent is an accession of Cruciferae genetic stocks from the Laboratory of Plant Breeding, Tohoku University, Japan. Two *R. sativus* L. cv. ‘Shogoin’ and ‘Risou’ are stocks of the Laboratory of Plant Breeding, Utsunomiya University, Japan.

Production of monosomic addition lines (MALs)

The schematic diagram for breeding of alloplasmic and autoplasmic MALs was shown in Fig. 1. Two *Raphanobrassica* and *B. napus* strain ‘N-350’ were grown in pots in a greenhouse. The hybridizations between two *Raphanobrassica* and *B. napus* strain ‘N-350’ were per-

formed using the conventional crossing method. Flower buds were emasculated one day before anthesis, immediately pollinated with fresh pollen and then bagged for about a week. To induce octoploidy, 0.2% colchicine solution was applied with small cotton plugs to the apical meristem of F_1 seedlings. To obtain the backcross progenies, F_1 plants were successively backcrossed with *B. napus* strain ‘N-350’ using the conventional crossing method. Embryo culture was also made for embryo rescue in some cases, according to the method of Bang *et al.* (1996). The somatic chromosome numbers of F_1 plants and their backcross progenies were counted using the Feulgen stain squash method followed by 1% acetocarmine staining.

For alloplasmic line, BC_2 plants were produced from 2 BC_1 plants with $2n=47$ and 52, and BC_3 plants were obtained from 5 BC_2 plants with $2n=39$ to 43 by backcrossing to *B. napus* strain ‘N-350’. Autoplasmic BC_2 and BC_3 plants were produced from 3 BC_1 plants with $2n=47$ and 48 and 5 BC_2 plants with $2n=40$ to 42 by backcrossing to *B. napus* strain ‘N-350’, respectively.

To classify the plants into nine types of MALs corresponding to R-genome, all the plants with $2n=39$ were first analyzed by the RAPD specific markers for each chromosome of R-genome in the BC_3 generation, and were successfully maintained as distinctive MALs in the BC_4 or BC_5 generation through successive backcrosses to *B. napus* strain ‘N-350’.

RAPD analysis

Polyacrylamide gel electrophoresis was employed according to Kaneko *et al.* (2001) with some modifications.

Fig. 1. The schematic diagram for breeding of alloplasmic and autoplasmic MALs. *Raphanobrassica* ‘RA-89’ was developed by hybridization of *R. sativus* L. cv. ‘Risou’ ($2n=18$, RR) and *B. rapa* L. ssp. *Japonica* cv. ‘Kyo-mizuna’ ($2n=20$, AA) (Matsuzawa *et al.* 2000). *Raphanobrassica* ‘Rb-63’ was developed from crossing between *R. sativus* L. cv. ‘Shogoin’ ($2n=18$, RR) and *B. oleracea* L. var. *acephala* cv. ‘Murasaki-habotan’ ($2n=18$, CC) (Matsuzawa *et al.* 1985).

Total DNA was isolated from young leaves using CTAB method. Reaction mixture (10 µl) contained 4 ng of genomic DNA, 0.2 µM of primer, 0.5 mM dNTPs (TOYOBO, Osaka, Japan), 1 × reaction buffer (Promega, Madison, WI, USA), and 1 unit of *Taq* polymerase (Promega) were pipetted into 0.2 ml tubes, and PCR was performed using Program Temp Control system PC-320 (ASTECC Co., Fukuoka, Japan). After an initial 5-min denaturation step at 94°C, fragments of DNA were amplified for 45 cycles (94°C for 30 sec, 35°C for 30 sec, 72°C for 1 min) with the extension step of the last cycle extended to 10 min. The amplified products were mixed with loading dye and loaded on a 0.5% polyacrylamide gel prepared in 1 × TBE buffer. After electrophoresis, the gel was stained with ethidium bromide and viewed with a UV illuminator.

The primers which yield R-genome specific markers were selected from the 400 12-mer sequences (Common A to D, BEX, Tokyo, Japan). The consistent markers observed in both *Raphanobrassica* and *R. sativus* but not observed in *B. napus* strain 'N-350' were assigned to R-genome specific RAPD markers. Using these RAPD markers, those specific to each radish chromosome of nine MALs were identified in the total of 14 BC₂ plants with 2n=39 to 43 in alloplasmic line. For autoplasmic line, more than 4 markers corresponding to each radish chromosome were selected from each MAL of alloplasmic line and used for identification of added radish chromosomes. These procedures were repeated twice.

Chromosome identification by GISH

Genomic *in situ* hybridization was performed with somatic cells of the 2n=39 plants, *R. sativus*, *B. napus* and *Raphanobrassica*. Chromosome preparation was performed using enzymatic maceration/air-drying method (Fukui 1996) with some modifications. Briefly, excised roots were pre-treated with 8-hydroxyquinoline (2 mM aqueous solution) at room temperature for 2 h, thereafter at 4°C for 2 h, and fixed in 3 : 1 ethanol: acetic acid for 24 h before being stored in 70% ethanol at –20°C. Fixed root tips were washed two times in 1 × enzyme buffer (10 min each) and digested enzymatically for 90 min at 37°C in a mixture comprising 2% cellulose 'Onozuka R-10' (Serva, Heidelberg, Germany) and 20% pectolyase Y-23 (SIGMA, St. Louis, MO, USA) in 1 × enzyme buffer. After a short rinse in 1 × enzyme buffer, the root tips were squashed in a drop of 3 : 1 ethanol: acetic acid. The coverslip then were removed by freezing, and the preparations were dehydrated by air drying.

Labeling of the DNA probes and genomic *in situ* hybridization were carried out according to the methods described in Schwarzacher *et al.* (1994) with some modifications. In short, genomic DNA isolated from *R. sativus* cv. 'Risou' was labeled with Tetramethyl-rhodamine-5-dUTP (Roche, Mannheim, Germany) by nick translation following the protocol provided by the manufacture (Roche).

Chromosome preparations were pre-treated with DNase-free RNase (100 µg/ml) in 2 × SSC for 1 h at 37°C, washed in 2 × SSC for 15 min, post-fixed with formaldehyde (4% aque-

ous solution) for 10 min, washed in 2 × SSC for 15 min, and dehydrated in an ethanol dilution series before being air-dried. The hybridization mixture consisted of 50% de-ionized formamide, 10% dextran sulphate, 2 × SSC and 0.25% SDS, and probe DNA was added to a concentration of 20 ng per slide. As a blocking DNA, genomic DNA isolated from *B. napus* strain 'N-350' was also mixed to a concentration of 400 ng per slide. The hybridization mixture was denatured for 10 min at 70°C, the slides were denatured together for 5 min at 80°C and the probes hybridized for 16 h in a humid chamber at 37°C. Slides were washed stringently with 2 × SSC for 10 min at 42°C and then with 0.2 × SSC for 5 min at 42°C. Finally, preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 2.5 µg/ml of 4',6-diamidino-2-phenylindole (DAPI; Serva).

Images were acquired with a computer-assisted cooled charge CCD camera (AxioCam MRm, Zeiss, Jena, Germany) attached to Zeiss Axio vert 200M epifluorescence microscope with the system software (Axio Vision Release 4.5, Zeiss), and retouched using Adobe Photoshop 7.0.

Characteristics of alloplasmic and autoplasmic MALs and transmission rate of the added alien chromosome

To characterize each type of alloplasmic and autoplasmic MALs, morphological, physiological, and cytogenetical characteristics were examined in the BC₄ and/or BC₅ generations. Germination rate was calculated with approx. 50 seeds of each type 1 week after sowing. Measurement of petal length and verification of flower malformation were made with ten flowers in full bloom. Pod length was measured with twenty pods 4 weeks after crossing. Meiotic chromosome configuration was examined at metaphase I (MI) in pollen mother cells (PMCs) using the 1% acetic orcein smear method. The ratio of pollen tetrads was assessed by observing about 100 cells stained with 1% acetic orcein. Pollen fertility was verified by examining 500 pollen grains from three flowers in full bloom using 1% acetocarmine staining method. Seed fertility was examined by calculating the ratio of seeds setting per pod developed from ca. 50 flowers when the MAL plants were backcrossed with rape.

Using RAPD markers specific to each chromosome, transmission of the added chromosome in each type of MALs through female gametes was rated with ca. 50 individuals from each type in the BC₄ and/or BC₅ generations.

Results

Development and identification of alloplasmic and autoplasmic MALs

In the hybridizations between two *Raphanobrassica* and rape (*B. napus*, strain 'N-350'), 27 and 19 F₁ hybrids were produced in the crosses of 'RA-89' × rape and rape × 'Rb-63', respectively. In the cross of 'RA-89' × rape, the somatic chromosomes of 21 F₁ hybrids were 2n=38, and their genome constitutions were assumed to be "RAAC". On the other hand, 11 F₁ hybrids obtained in the crosses of rape ×

Table 1. Distribution of somatic chromosome numbers in BC₁, BC₂ and BC₃ plants derived from both alloplasmic F₁ plant (*Raphanobrassica* 'RA-89' × rape, 2n=76) and autoplasmic F₁ plant (rape × *Raphanobrassica* 'Rb-63', 2n=74) after successive backcrosses to *B. napus* strain 'N-350'

Line	No. of plants Observed	Somatic chromosome number (2n)																					
		<38	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	—	57	57<
<alloplasmic>																							
BC ₁	95 ^a	1	1		1		2	3	6	5	5	9	6	4	10	14	13	6	2	1		1	5
	(%)	1.1	1.1		1.1		2.1	3.2	6.3	5.3	5.3	9.5	6.3	4.2	10.5	14.7	13.7	6.3	2.1	1.1		1.1	5.3
BC ₂ ^b	83	7	10	17	14	13	9	8	4				1										
	(%)	8.4	12.0	20.5	16.9	15.7	10.8	9.6	4.8				1.2										
BC ₃ ^c	190	22	59	50	28	19	7	1	2						1								1
	(%)	11.6	31.1	26.3	14.7	10.0	3.7	0.5	1.1						0.5								0.5
<autoplasmic>																							
BC ₁	25									2		6	3	1		4	2	1	2			3	1
	(%)									8.0		24.0	12.0	4.0		16.0	8.0	4.0	8.0			12.0	4.0
BC ₂ ^d	62		1	2	7	7	14	18	5	5	2	1											
	(%)		1.6	3.2	11.3	11.3	22.6	29.0	8.1	8.1	3.2	1.6											
BC ₃ ^e	181		50	69	43	16	3																
	(%)		27.6	38.1	23.8	8.8	1.7																

^a In total 95 BC₁ plants, 78 were obtained by backcross with *B. napus* strain 'N-350' following to embryo culture.

^b BC₂ plants were obtained from 2 BC₁ plants with 2n=47 and 52 by backcrossing to *B. napus* strain 'N-350'.

^c BC₃ plants were obtained from 5 BC₂ plants with 2n=39 to 43 by backcrossing to *B. napus* strain 'N-350'.

^d BC₂ plants were obtained from 3 BC₁ plants with 2n=47 and 48 by backcrossing to *B. napus* strain 'N-350'.

^e BC₃ plants were obtained from 5 BC₂ plants with 2n=40 to 42 by backcrossing to *B. napus* strain 'N-350'.

'Rb-63' were 2n=37, with assumed genome of "ACRC". These F₁ hybrids obtained in each cross-combination exhibited morphological characteristics that are intermediate between their parents. The F₁ hybrids produced abortive pollens and no seeds were obtained by the crossing with rape. Each allooctoploid line obtained from two cross-combinations by colchicine treatment was repeatedly backcrossed to rape, producing BC₁, BC₂ and BC₃ plants. Somatic chromosome numbers of these progenies were shown in Table 1. In alloplasmic line, the seed setting of 2 BC₁ plants (2n=47 and 52) which were used to produce BC₂ plants was 6.9 and 3.4 seeds, respectively, after backcrossing with rape. From the morphological and cytogenetical observations, these materials were assumed to be the AACC genome constitution added 1 R-genome with some alien chromosomes. In BC₃ generation, the 2n=39 plants were progressively obtained. The number of plants obtained were 50 (26.3%) in alloplasmic lines and 69 (38.1%) in autoplasmic lines, although they were first obtained in BC₂ generation.

In the profiles of GISH, 9 signals were observed in somatic chromosome of radish, whereas no signal was detected in rape (date not shown). Eighteen radish signals were observed in somatic metaphase chromosome of *Raphanobrassica* (Fig. 2A), and a single large radish signal was observed in the 2n=39 plant (Fig. 2B). This result confirmed that the radish chromosome derived from *Raphanobrassica* was substantially transferred to the backcross progenies. On the other hands, a lot of small signals were also detected in the somatic cell of 2n=39 plant. These small signals may reflect the homoeologous regions to R-genome included in A- and C-genomes of rape.

After examining a total of 400 RAPD primers for prelim-

Fig. 2. Genomic *in situ* hybridization of radish total DNA (red) probes to somatic metaphase chromosomes of *Raphanobrassica* (A) and the 2n=39 plant (B). The chromosomes are counter-stained with DAPI (blue). Arrow indicates radish chromosome.

inary classification of MALs, 232 markers specific to R-genome were detected from 99 primers. Of these 175 markers could be classified tentatively into 8 types (a–i, except for the h-type) according to their segregation patterns (Table 2). The number of types for R-genome specific markers in the 14 BC₂ plants was close agreement with the number of added radish chromosomes expected from somatic chromosome number of these plants. Therefore, these 8 types may correspond to each of 8 chromosomes of the R-genome, and these markers specific to each type may be useful for the identification of radish chromosomes in further crosses with rape.

On the other hands, 8 types similar to alloplasmic line were detected in the 10 BC₂ plants of autoplasmic line, and 10 R-genome specific markers that had not detected in alloplasmic line were newly detected in 2 BC₂ plants (No. 1 and 10) (Table 3). These 10 markers were assumed to correspond

Table 2. Identification of the added radish chromosomes by RAPD specific markers in BC₂ plants (2n=39~43) of alloplasmic (radish cytoplasm) line

Type	Sample No.														Total	Specific markers
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
a	+	+		+					+	+					5	42
b										+					1	19
c	+									+					2	19
d		+	+	+				+	+			+		+	7	28
e				+	+				+					+	4	25
f	+														1	16
g		+											+	+	3	11
h															–	–
i	+	+	+		+	+	+		+	+	+		+	+	11	15
Total	4	4	2	3	2	1	1	1	4	4	1	1	2	4		175
Ex ^a	3	2	1	1	2	2	1	1	3	5	1	1	2	2		
2n ^b	41	40	39	39	40	40	39	39	41	43	39	39	40	40		

^a Number of radish chromosomes expected from somatic chromosome number.^b Somatic chromosome number.**Table 3.** Identification of the added radish chromosomes by RAPD specific markers in BC₂ plants (2n=39~41) of autoplasmic (rape cytoplasm) line

Type	Sample No.										Total	List of specific markers	
	1	2	3	4	5	6	7	8	9	10			
a				+							1	10 ^a	A12 ₅₁₀ , A48 ₃₆₀ , B01 ₂₉₀ , B04 ₆₂₀ , B15 ₆₂₀ , B35 ₆₇₀ , B35 ₅₂₀ , C40 ₈₂₀ , C40 ₃₄₀ , D60 ₂₀₀
b	+		+				+				3	5	A09 ₄₂₀ , A09 ₁₉₀ , A12 ₆₂₀ , D25 ₁₉₀ , D76 ₂₂₀
c				+		+		+			3	4	A26 ₈₂₀ , A94 ₆₂₀ , B42 ₅₁₀ , B88 ₂₀₀
d								+	+		2	6	A26 ₄₇₀ , A57 ₆₂₀ , B15 ₄₇₀ , B35 ₇₇₀ , B42 ₃₀₀ , B96 ₂₀₄₀
e		+		+	+	+	+			+	6	4	A98 ₅₂₀ , B88 ₄₄₀ , D72 ₃₀₀ , D94 ₂₀₀
f						+	+			+	3	4	A13 ₂₈₀ , A66 ₄₇₀ , C30 ₄₇₀ , C79 ₄₇₀
g				+	+			+			3	4	A39 ₇₇₀ , A39 ₆₂₀ , A83 ₈₂₀ , D07 ₄₇₀
h	+									+	2	10	A14 ₃₀₀ , A14 ₁₇₀ , A35 ₅₂₀ , A37 ₂₈₀ , A39 ₅₂₀ , A39 ₃₀₀ , B01 ₄₄₀ , B01 ₃₄₀ , B32 ₃₄₀ , C30 ₄₂₀
i			+		+					+	3	4	B08 ₅₁₀ , B15 ₃₂₀ , B55 ₃₂₀ , C30 ₃₂₀
Total	2	1	2	4	3	3	3	3	1	4	–	51	
Ex ^b	2	1	3	2	2	3	3	2	1	2			
2n ^c	40	39	41	40	40	41	41	40	39	40			

^a Number of used and detected specific markers.^b Number of radish chromosomes expected from somatic chromosome number.^c Somatic chromosome number.

to the remaining one chromosome of the R-genome and designated to 'h-type'.

Thus, from RAPD analysis, added radish chromosome composition in alloplasmic and autoplasmic BC₂ progenies was established by each nine chromosome specific markers. By successive backcrosses for these BC₂ progenies with rape and identification of radish chromosome using each chromosome specific RAPD markers, alloplasmic MAL with 8 types except for the h-type and autoplasmic MAL with complete 9 types were produced in BC₃ and BC₄ generations.

Characteristics of the MALs

Characteristics of alloplasmic and autoplasmic MALs were shown in Table 4. Germination rate varied among the

types, ranging from "normal" (a-, c-, e-, f-, g-type) to "middle" (b-, h-, i-type) and "low" (d-type). The d-type showed a distinctly low germination rate in both alloplasmic and autoplasmic MALs. In alloplasmic MAL, most types had malformed flowers (antherless, petaloid, or stunted petals) (Fig. 3A–C), whereas all of the nine types in autoplasmic MAL produced normal flowers (Fig. 3D–F). The flowers of alloplasmic MAL were smaller than those of autoplasmic MAL. All the types of alloplasmic and autoplasmic MALs except for the g-type produced yellow flowers like rape. On the other hand, flowers of the g-type from both alloplasmic and autoplasmic MALs were characterized by their white color like radish. No morphological differences were recognized in their leaves among the MALs (date not shown).

All types of autoplasmic MAL predominantly exhibited

Table 4. Morphological, physiological and cytogenetical characteristics of alloplasmic and autoplasmic MALs in BC₃ to BC₅ generations in the comparison with two radish cultivar 'Shogoin', 'Risou', two *Raphanobrassica* 'RA-89', 'Rb-63' and rape strain 'N-350'

Character	Nine types of two MALs																		Radish		Raphanobrassica		Rape	
	a		b		c		d		e		f		g		h		i							
	allo ^a	auto ^b	allo	auto	allo	auto	allo	auto	allo	auto	allo	auto	allo	auto	allo	auto	allo	auto	Risou	Shogoin	RA-89	Rb-63	N-350	
No. of plants observed	12	1	11	11	5	4	4	1	8	7	1	2	2	2	10	6	6	5	5	5	5	5	5	
Germination rate (%)	98.0	94.0	62.0	98.0	92.0	92.0	44.0	61.4	96.0	100.0	96.0	100.0	96.0	96.0	88.0	72.0	78.0	100.0	100.0	70.0	50.0	100.0	100.0	
Flower																								
malformation	— ^c	—	+ ^d	—	+	—	+	—	+	—	+	—	+	—	—	+	—	—	—	—	—	—	—	
Petal length (mm)	15.5	14.7	13.3	15.7	16.0	17.9	12.9	16.1	15.3	15.6	12.3	17.9	13.2	14.8	14.4	13.6	18.7	22.7	21.9	20.1	24.8	16.0	16.0	
Petal color ^e	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	W	W	Y	Y	Y	W	W	W	W	Y	Y	
Pod length (mm)	40.3	56.8	30.6	60.2	44.4	51.7	46.9	66.3	35.9	54.9	34.8	73.2	34.1	52.9	27.5	31.5	59.8	47.4	55.9	48.9	55.8	57.8	57.8	
Chromosome pairing at MI of PMCs	no ^f	19 _{II} +1 _I	no	19 _{II} +1 _I	no	19 _{II} +1 _I	no	19 _{II} +1 _I	no	19 _{II} +1 _I	no	19 _{II} +1 _I	no	19 _{II} +1 _I	19 _{II} +1 _I	no	19 _{II} +1 _I	no	no	no	no	no	no	
Pollen tetrad (%)	no	97.3	no	92.3	no	98.0	no	96.5	no	94.6	no	99.2	no	98.6	97.1	no	100.0	no	no	no	no	no	no	
Pollen fertility (%)	88.0	87.9	ms ^g	82.9	ms	83.0	ms	87.2	ms	47.6	ms	88.1	ms	89.5	79.5	ms	84.9	93.4	94.8	62.6	85.9	95.9	95.9	
Seed setting (grain)	11.8	12.8	3.8	9.7	13.8	5.8	1.1	0.3	4.4	9.5	7.0	14.2	6.3	10.4	4.5	2.6	6.2	2.3	2.6	1.3	4.3	16.7	16.7	
1000 seed weight (g)	4.38	4.76	4.64	4.26	5.20	4.86	2.44	2.20	4.32	4.30	3.28	4.10	5.05	4.97	4.04	3.42	4.52	20.48	14.96	3.12	5.84	4.84	4.84	

^a alloplasmic MAL carrying radish cytoplasm.^b autoplasmic MAL carrying rape cytoplasm.^c normal flowers with six normal anthers which producing abundant pollen.^d abnormal flowers such as antherless, petaloid and stunted petals.^e Y: yellow, W: white.^f Not observed.^g male sterility.

chromosome configuration of 19_{II}+1_I at MI of PMCs, and pollen tetrad ranged from 92.3% (b-type) to 100.0% (i-type). In autoplasmic MAL carrying the cytoplasm of rape, all of the nine types had a longer pod length than those of alloplasmic MAL although the h-type had a short pod (28 mm). In contrast, most of alloplasmic MAL having the radish cytoplasm showed short pod length and male sterility, and only the a-type produced normal flowers and had a high pollen fertility (88.0%). Seed setting of the MALs in both alloplasmic and autoplasmic lines varied from 1.1 (d-type) to 13.8 (c-type) and from 0.3 (d-type) to 14.2 (f-type), respectively. The d-type from both lines showed a low seed setting and produced small seeds.

Transmission rate of the added radish chromosome

The calculated transmission rates in alloplasmic MALs varied from 11.6% for the g-type to 93.5% for the e-type, whereas those in autoplasmic MALs varied from 11.1% for the g-type to 100.0% for the d-type (Table 5). The d-type showed a high transmission rate in both MALs, whereas the g-type had a low transmission rate in both lines. These results might suggest that each radish chromosome added to the genetic background of rape was certainly transferred to the next generations via female gamete, although large differences were observed in the transmission rate of individual radish chromosomes.

Discussion

Dolstra (1982) has reported that productions of both F₁ hybrids and backcross progenies were difficult in direct hybridization between radish and rape due to their poor

crossability. However, F₁ hybrids were produced stably by using *Raphanobrassica* as the bridging plants in this study. This fact was also confirmed by Peterka *et al.* (2004). From these results, it was suggested that the amphidiploids such as *Raphanobrassica* or *Brassicoraphanus* are valuable to overcome the poor crossability between radish and rape. Khush and Brar (1992) reported that the crosses between the species with different ploidy levels are difficult to achieve, and the integration of ploidy levels in parent species may help overcome the hybridization barrier in these cross combinations. However, F₁ plants (RAAC, 2n=38; and ACRC, 2n=37) showed extremely low pollen fertility and produced only a few progeny seeds even by open pollination (date not shown). So, the colchicine treatment was conducted for F₁ plants to improve both pollen and seed fertility by chromosome doubling. As a result, a large number of seeds were obtained from allooctaploid F₁ plants (RRAAAACC and AACCRRC) by backcrossing to *B. napus* to produce BC₁ plants. The chromosome number of BC₁ plants principally showed a tendency to be allopolyploid (2n=47±α) and was lower than the assumed chromosome number of 2n=57 and 56 (RAAACC and AACCRRC), and seed fertility of BC₁ plants were improved. From these BC₁ plants (2n=47 to 52), the BC₂ plants having the nuclear background of rape (AACC) with some alien chromosomes were obtained (Table 1). Moreover, in this study, we could bred both alloplasmic and autoplasmic MALs through only three or four time backcrosses with rape by application of *Raphanobrassica* (RRAA and RRCC) which is genetically-stable and maintained for a long term. Other MAL series were also effectively bred by using *Raphanobrassica* (Kaneko *et al.* 1987, 2001). Therefore, it is also suggested that the application of

stable *Raphanobrassica* may be effective for the reduction of breeding term of MAL.

For chromosome identification, nine types of R-genome specific RAPD markers corresponding to each of the nine chromosomes of radish were detected using the BC₂ plants of both alloplasmic and autoplasmic lines and has been used in this study (Table 2 and Table 3). However, the number of types classified by each chromosome specific RAPD markers was occasionally inconsistent with the number of added radish chromosomes expected from somatic chromosome number in some plants of BC₂ progenies. This discordance was observed also in the production of potato-tomato MALs (Garriga-Calderé *et al.* 1997) and rape-radish MALs (Peterka *et al.* 2004), and may be due to the chromosome substitution or translocation between a radish chromosome and A- or C-genome chromosome of rape in this study. It was also suggested that the chromosomes of A- or C-genome of *Raphanobrassica* (RRAA or RRCC) were supplementally added with one of R-genome in these BC₂ progenies. By GISH analysis, it was clarified that the added chromosome for $2n=39$ plants is derived not from A- or C-genome but from R-genome of *Raphanobrassica* (Fig. 2B). Furthermore, in the cytogenetic observation for autoplasmic MAL, all of the nine types predominantly showed the chromosome configuration of $19\text{ II} + 1\text{ I}$ (Table 4). The *R. sativus*–*B. oleracea* MALs ($2n=19$) and the *R. sativus*–*B. rapa* MALs ($2n=19$) exhibited the chromosome configuration of $9\text{ II} + 1\text{ I}$ at meiosis (Kaneko *et al.* 1987, 2001). These results were suggested that the chromosome of R-genome of radish was extremely rare syndesis with the chromosome of A- or C-genome in the genetic background of rape (AACC). Accordingly, the chromosomal configuration of $19\text{ II} + 1\text{ I}$ in this study would result from complete synapsis to the homologous chromosomes of rape plus the added chromosome of the R-genome at meiosis. From these results of RAPD, GISH and cytogenetic analyses of MAL, it was confirmed that the 8-types of alloplasmic MAL and the complete 9-types of autoplasmic MAL were true MALs of alloplasmic (radish cytoplasm) and autoplasmic (rape cytoplasm) *B. napus*.

Investigation of morphological and physiological traits of alloplasmic and autoplasmic MALs and comparative studies of these MALs may be useful not only for analysis of gene(s) located on each chromosome, but also for analysis the interrelationship between cytoplasm and chromosomes of donor plant (Budahn *et al.* 2008). In alloplasmic line carrying the cytoplasm of radish, all of 8 types except for a-type produced the malformed flowers such as antherless or petaloid flowers and those with stunted petals (Fig. 3B, C and Table 4). Such phenomena have been observed in the progenies of intergeneric hybridization between *R. sativus* and *B. napus* (Paulmann and Röbbelen 1988). Attia and Röbbelen (1986) proposed that some of these disorders for fertility have been attributed to errors in the genetic control of meiotic chromosome configuration that is stabilized after repeated backcrosses to rape. Meanwhile, the disomic addition line

Fig. 3. Morphological features of flower in some types of alloplasmic and autoplasmic MALs; alloplasmic line (A–C) and autoplasmic line (D–F), and their parental species (G–I). Three types of MALs: a-type (A and D), d-type (B and E), and g-type (C and F). Parental species: *B. napus* strain ‘N-350’ (G), *Raphanobrassica* ‘Rb-63’ (H), and *R. sativus* cv. ‘Shogoin’ (I).

(DAL) plants having a pair of radish chromosomes restored pollen fertility in the progenies of the somatic hybrids derived from protoplast fusion between *B. napus* and *R. sativus* with cytoplasmic male sterility (CMS) (Sakai and Imamura 2003). Bang *et al.* (2002) reported that some types (a, b, c, d, e, f, k and l) in the *R. sativus*–*M. arvensis* MAL ($2n=19$) restored male fertility, ranging from 3.4% to 85.6% in alloplasmic (*Moricandia* cytoplasm) radish. Budahn *et al.* (2008) also reported that only one type (designated as “f-type” in their terminology) in the alloplasmic (radish cytoplasm) *B. napus*–*R. sativus* MAL maintained male fertility. In this study, only the a-type in the alloplasmic (radish cytoplasm) MAL showed normal flowers and restored pollen fertility (88.0%, Fig. 3A and Table 4) although all of the nine types in autoplasmic line produced normal flowers. Moreover, the meiotic chromosome configuration at MI in PMCs of autoplasmic MAL was $19\text{ II} + 1\text{ I}$ and pollen tetrad (%) showed high rate (Table 4), in contrast to what was observed

Table 5. Female transmission rate of each radish chromosome in the alloplasmic and autoplasmic MALs (a- to i-type, 2n=39)

Line	Type	Generations	No. of plants observed	Transmission rate of MAL (%)
<alloplasmic>	a	BC ₄ , BC ₅ ^a	32, 42 ^b	53.6±18.5
	b	BC ₅	49	42.9
	c	BC ₅	47	34.0
	d	BC ₄	36	72.2
	e	BC ₄ , BC ₅	37, 47	93.5±0.2
	f	BC ₄	68	14.7
	g	BC ₄ , BC ₅	67, 152	11.6±11.7
	i	BC ₄ , BC ₅	17, 86	36.3±6.9
<autoplasmic>	a	BC ₄	40	50.0
	b	BC ₄	42	31.0
	c	BC ₄	42	28.6
	d	BC ₃	21	100.0
	e	BC ₄	45	60.0
	f	BC ₃	49	30.6
	g	BC ₄	45	11.1
	h	BC ₄	42	33.3
	i	BC ₄	46	28.3

^a the transmission rate was calculated in both BC₄ and BC₅ generations.

^b the number of left and right are the number of plants observed in BC₄ and BC₅ generations, respectively.

by Paulmann and Röbbelen (1988). Therefore, it was suggested that the male sterility observed in this study was induced by the radish cytoplasm but not by the aberrancy in chromosome configuration at meiosis, and the fertility restoring gene(s) for male sterility may be located on the a-chromosome of radish. It was also assumed that the a-type in this study and the f-type in previous study by Budahn *et al.* (2008) would be having the same chromosome of radish. Therefore, comparative studies of these two strains of *B. napus*-*R. sativus* MAL may provide further information to understand the restore gene(s) for cytoplasmic male sterility in radish.

The g-types from both alloplasmic and autoplasmic lines were characterized by the distinctive white flowers similar to those of radish (Fig. 3C, F and Table 4). Kato and Tokumasu (1976) reported that a single dominant gene controlled white petal color in radish, and Sernyk and Stefansson (1982) reported that the *B. napus* strain added with a *R. sativus* chromosome developed at University of Manitoba produced white flowers. Therefore, it was assumed that a single dominant gene determining white petal color was located on the g-chromosome of radish. The g-type may trigger a study on the expression mechanism of white color pigmentation in Brassicaceae.

Germination rate, seed fertility and transmission rate are important factors for maintenance of MAL. In alloplasmic and autoplasmic lines, all of the types except for the d-type showed high germination rate and seed fertility (Table 4). Therefore, these MALs except for the d-type would be able to maintain their progenies by backcrossing with rape. On

the other hands, the d-type of both alloplasmic and autoplasmic lines showed both low germination rate and extremely low seed fertility and produced small seeds. Some hybridization barriers inducing hybrid inviability, weakness and breakdown (Khush and Brar 1992) were assumed to exhibit these erosions by traits on the d-chromosome of radish. Moreover, the large differences in the transmission rate of individual radish chromosomes are consistent with similar reports for MALs from other *Brassica* species (Kaneko *et al.* 1987, 2001, Bang *et al.* 2002, Budahn *et al.* 2008). For the types with low transmission rate, selected smaller seeds (SSS) method may be effective for the acquisition of MALs and providing the valuable MAL plants for genetic research (Kaneko *et al.* 2003).

Several MAL series have been used to analyze for some agronomic traits and gene(s) located on the added chromosome, for example disease resistance to blackleg (*Leptosphaeria maculans*) (Chèvre *et al.* 1996), turnip mosaic virus (TuMV) (Kaneko *et al.* 1996), beet cyst nematode (Peterka *et al.* 2004) and photorespiratory characteristics (Bang *et al.* 2009). The alloplasmic and autoplasmic MALs produced in this study are useful to explore agronomic traits locating on each chromosome of radish as well as to introgress the promising disease resistance gene(s) of radish to *B. napus*. Japanese radish cultivar 'Shogoin', which was used as the chromosome donor plant of autoplasmic MAL, shows complete clubroot resistance (Ashizawa *et al.* 1980). The evaluation of clubroot resistance of this MAL may be useful to examine the resistance mechanism of clubroot in radish.

Recently, Budahn *et al.* (2008) reported that they produced another *B. napus*-*R. sativus* MAL possessing radish chromosomes derived from fodder and/or oil radish. The MALs produced in this study are different from those of Budahn *et al.* (2008), since our MALs possess each of nine chromosomes derived from Japanese radish cultivars ('Risou' and 'Shogoin'). Our MALs, therefore, may provide more important information for the modification of fresh-radish cultivars in East Asia.

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