Production of Intergeneric Hybrids between the C₃-C₄ Intermediate Species *Diplotaxis tenuifolia* (L.) DC. and *Raphanus sativus* L.

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In intergeneric crossings between *Diplotaxis tenuifolia* (2n = 22, DtDt) and five cultivars of *Raphanus sativus* (2n = 18, RR), an intergeneric F₁ hybrid was produced from the crossing of *D. tenuifolia* × *R. sativus* cv. '4-season leaf' through ovary culture followed by embryo culture. The induced amphidiploid (2n = 40, DtDtRR) showed well-regulated meiotic features at PMCs and a high pollen fertility (75%). Three BC₁ hybrids with DtRR (2n = 29) or DtDtR (2n = 31) genome constitutions were obtained by the same embryo rescue procedure in the crossings of amphidiploid × *R. sativus* and *D. tenuifolia* × amphidiploid, respectively. In the successive backcrossings of two BC₁ hybrids (DtRR, 2n = 29) to *R. sativus*, 102 BC₂ hybrids were obtained by conventional pollination. In the reciprocal crossing of *R. sativus* × BC₁ hybrids, 12 reciprocal BC₂ hybrids were also produced without embryo rescue. The somatic chromosome number of 89 BC₂ hybrids with *D. tenuifolia* cytoplasm and 12 reciprocal BC₂ hybrids with *R. sativus* cytoplasm ranged from 2n = 18 to 2n = 23 that were estimated to carry 2n = 18 chromosomes of *R. sativus* and zero to five chromosomes of *D. tenuifolia*. Among them, 24.7% of the BC₂ hybrids and 41.6% of the reciprocal BC₂ hybrids were assumed to be monosomic addition lines (MALs, 2n = 19). The novel intergeneric hybrids obtained in this study could become useful materials for investigating the genetic effects on C₃-C₄ intermediate traits at the genomic and chromosomal levels, as well as for estimating the performance of genetic improvement in Brassicaceae.

Key Words: *Diplotaxis tenuifolia*, *Raphanus sativus*, C₃-C₄ intermediate species, intergeneric hybrids, ovary and embryo culture.

Introduction

Diplotaxis tenuifolia (L.) DC. is a C₃-C₄ intermediate species that is characterized by a high concentration of mitochondria and chloroplasts in the bundle-sheath cells and a high potential for reassimilation of photorespired CO₂ (Apel *et al.* 1996, 1997). In Brassicaceae, other C₃-C₄ intermediate plants were identified within species of the genera *Moricandia*, *Diplotaxis* and *Brassica* (Apel *et al.* 1997). The C₃-C₄ intermediate traits, especially low photorespiration activity, may be valuable for the breeding of cultivated crops in Brassicaceae.

M. arvensis which was first reported as a C₃-C₄ intermediate species has attracted breeders' attention to introgress C₃-C₄ intermediate traits into cultivated crops. A number of intergeneric hybrids between *M. arvensis* and some cultivated crops have been produced by the application of conventional pollination as well as embryo rescue technique and protoplast fusion (Toriyama *et al.* 1987, Takahata 1990, Takahata and Takeda 1990, Takahata *et al.* 1993, Kirti *et al.* 1992, Razmjoo *et al.* 1996, Bang *et al.* 1996a). Their progeny, however, could hardly be obtained due to intrinsic cross incompatibility in the successive backcrossings to the cultivated crops. Bang *et al.* (1996a, 2002) developed F₁ hybrids by overcoming the barriers in the intergeneric crossing between *M. arvensis* and *R. sativus*, and then induced their backcrossed progenies by the embryo rescue technique followed by chromosome doubling of the F₁ hybrids. Moreover, they bred twelve types of *M. arvensis* monosomic addition lines (MALs) of alloplasmic (*M. arvensis*) *R. sativus* in the following generation.

The C₃-C₄ intermediate species in the Brassicaceae including *M. arvensis* and *D. tenuifolia* belong to the same "Rapa/Oleracea" lineage at the level of molecular phylogeny using chloroplast DNA restriction site variation (Warwick *et al.* 1992, Warwick and Black 1994). These results indicate the existence of a common phylogenetic ancestor and monophyletic evolution of the C₃-C₄ intermediate traits in the Brassicaceae (Apel *et al.* 1997). However, the genetic divergence in the chloroplast genome does not necessarily reflect changes in the C₃-C₄ intermediate traits, based on nuclear genes. The intergeneric hybrids with various genome constitutions and MALs between two C₃-C₄ intermediate species and cultivated crops could provide more valuable information to understand the evolution and the genetic system of

the C₃-C₄ intermediate traits. Although intergeneric hybrids between *D. tenuifolia* and cultivated crops (*B. rapa*, *B. nigra*, *B. oleracea* and *Eruca sativa*) have been produced (Harberd and McArthur 1980, Takahata and Hinata 1983, Salisbury 1989), hybridization between *D. tenuifolia* and *R. sativus* has not been reported. In this study, some aspects of the intergeneric hybrids between *D. tenuifolia* (C₃-C₄ intermediate species) and *R. sativus* (C₃ species) will be described.

Materials and Methods

D. tenuifolia (L.) DC. (2n=22, DtDt) and five cultivars of *R. sativus* L. (2n=18, RR) were used as parents in reciprocal crossings. *D. tenuifolia* strain 1, an accession of Cruciferae Genetic Stocks in the Laboratory of Plant Breeding, Tohoku University, Japan, was kindly provided by former Professor K. Hinata. The *R. sativus* cultivars, such as 'Aokubi-Miyashige-Nagabuto', '4-season leaf', 'Chonggag-Altari', 'Pungmi-Altari' and 'Chung-pi Hong-sim' were selected from the accessions of the Laboratory of Plant Breeding, Utsunomiya University, Japan. Flower buds were emasculated one day before anthesis, immediately pollinated with fresh pollen and then bagged. Ten pistils were observed fluorometrically by 0.1% aniline blue staining 48 hours after pollination to detect pre-fertilization barriers. Pollen germination and pollen tube growth were determined based on the pollen germination index (PGI) according to the method of Matsuzawa (1983).

To obtain F₁ hybrids, ovary culture followed by embryo culture was employed when *D. tenuifolia* was used as the pistillate parent, while only embryo culture was used when *R. sativus* was used as the pistillate parent. These ovary and/or embryo cultures were performed according to the method of Bang *et al.* (1996b). The plantlets obtained were grown in pots after acclimatization in a plant growth room at about 15°C. To induce an amphidiploid, 0.2% colchicine solution was applied with small cotton plugs to the apical meristem of F₁ seedlings. Selfing of the induced amphidiploid and reciprocal backcrossings of the amphidiploid to parental spe-

cies, followed by embryo rescue were performed to obtain F₂ and BC₁ hybrids with various genome constitutions. BC₂ hybrids were produced from successive backcrossings of BC₁ hybrids to *R. sativus* and its reciprocal crossing without embryo rescue.

Somatic chromosomes were observed using the Feulgen stain squash method followed by 1% acetocarmine staining. Meiotic chromosomes were examined in pollen mother cells (PMCs) using the 1% acetic orcein smear method. Pollen fertility was determined by observing about 1000 pollen grains after staining with 1% acetocarmine.

Results

Production, morphology and cytological stability of F₁ hybrids

When *D. tenuifolia* was used as the pistillate parent, the pollen grains of *R. sativus* cv. 'Aokubi-Miyashige-Nagabuto' and '4-season leaf' germinated on the papillae of *D. tenuifolia* and then the pollen tubes elongated near the ovules, showing a mean PGI of 3.0 (Table 1). From these crossings, one and two embryos were developed through ovary culture, respectively. In the crossing of *D. tenuifolia* × '4-season leaf', one of the two embryos grew to a F₁ hybrid after embryo culture. The pollen grains of the other cultivars hardly germinated on the papillae of *D. tenuifolia*, from which no embryo was obtained. On the other hand, the pollen grains of *D. tenuifolia* hardly germinated on the papillae of any cultivars of *R. sativus*, with the mean PGI ranging from 1.6 to 2.4. No embryos were obtained from the crossing of *R. sativus* × *D. tenuifolia*.

An amphidiploid was induced from the F₁ hybrid by colchicine treatment. This amphidiploid (2n=40, DtDtRR) was intermediate between the parents for morphological characters (Fig. 1A, 1B and 1C) and showed well-regulated meiotic features in PMCs forming 20 bivalents (20II) at metaphase I (M I) and a 20 to 20 chromosome segregation at metaphase II (M II) (Table 2, Fig. 2A). Pollen fertility of the amphidiploid was 75.0%.

Table 1. Production of F₁ hybrid(s) in intergeneric crossings between *D. tenuifolia* and five cultivars of *R. sativus*

Cross combination	PGI ¹⁾	No. of flowers pollinated	No. of ovaries cultured	No. of embryos cultured	No. of F ₁ hybrid(s)
<i>D. tenuifolia</i> × 'Aokubi-Miyashige-Nagabuto'	3.0	60	50	1	0
r.c. ²⁾	2.3	120		0	—
<i>D. tenuifolia</i> × '4-season leaf'	3.0	110	100	2	1
r.c.	1.9	120		0	—
<i>D. tenuifolia</i> × 'Chonggag-Altari'	2.0	60	50	0	—
r.c.	2.4	200		0	—
<i>D. tenuifolia</i> × 'Pungmi-Altari'	2.1	60	50	0	—
r.c.	1.6	130		0	—
<i>D. tenuifolia</i> × 'Chung-pi Hong-sim'	2.3	60	50	0	—
r.c.	1.7	120		0	—

¹⁾ Pollen Germination Index according to Matsuzawa (1983), 0 ≤ PGI ≤ 4.

²⁾ Reciprocal crossing.

Table 2. Chromosome configurations at metaphase I of PMCs and pollen fertility in the amphidiploid and the BC₁ hybrids produced from intergeneric crossings between *D. tenuifolia* (2n=22, DtDt) and *R. sativus* cv. '4-season leaf' (2n=18, RR)

Hybrids	No. of plants observed	2n	Genome constitution	Mean chromosome configurations at M I			Pollen fertility (%)
				No. of cells observed	II	I	
Amphidiploid ¹⁾	1	40	DtDtRR	50	20.0	0.0	75.0
BC ₁ hybrid	2	29	DtRR	70	9.0	11.0	48.6
BC ₁ hybrid	1	31	DtDtR	20	11.0	9.0	14.8

¹⁾ Amphidiploid was obtained from the F₁ hybrid by colchicine treatment.

Production of F₂ and BC₁ hybrids and their morphological and cytological characteristics

Selfing of the amphidiploid and reciprocal crossings between the amphidiploid and parental species were performed to obtain hybrids with various genome constitutions (Table 3). Selfing did not enable to produce any progeny even through ovary culture because the pollen grains of the amphidiploid did not germinate on its own pistils.

In the crossing of amphidiploid × *R. sativus*, in 20% of the amphidiploid pistils, pollen tube elongation of *R. sativus* was observed and a few pollen tubes reached the ovules of the amphidiploid (Table 3). Six embryos were produced from 100 ovaries cultured, from which two BC₁ hybrids were obtained after embryo culture. Other four embryos degenerated during the embryo culture. No embryos were obtained through ovary culture in the reciprocal crossing of *R. sativus* × amphidiploid. The morphological characters of the BC₁ hybrids were more similar to those of *R. sativus* cv. '4-season leaf' than to those of *D. tenuifolia* in terms of plant shape and leaf division (Fig. 1D).

In the crossing of *D. tenuifolia* × amphidiploid, the pollen tubes of the amphidiploid elongated normally to the ovules of *D. tenuifolia*, while in the reciprocal crossing, only a few pollen tubes reached the ovules of the amphidiploid (Table 3). In the crossing of *D. tenuifolia* × amphidiploid, four embryos were produced from 90 cultured ovaries, from which a BC₁ hybrid was obtained after embryo culture. The BC₁ hybrid showed a closer similarity to *D. tenuifolia* than to *R. sativus* cv. '4-season leaf' in terms of plant shape, number of leaves, leaf color and leaf division (Fig. 1E). Other 3 plants were similar to the female parent not only in the morphological characters but also in the chromosome number of *D. tenuifolia* (data not presented).

Chromosome configuration at metaphase I of PMCs and pollen fertility were examined in three BC₁ hybrids (Table 2). Two BC₁ hybrids derived from the crossing of amphidiploid × *R. sativus* exhibited the chromosome configuration type of 9 bivalents and 11 univalents (9II+11I) at metaphase I in all the PMCs observed (Fig. 2B), where the genome constitution was assumed to be DtRR (2n=29).

Table 3. Production of F₂ and BC₁ hybrids in selfing and reciprocal backcrossings of amphidiploid, and BC₂ hybrids in reciprocal crossings between BC₁ hybrid (2n=29, DtRR) and *R. sativus* cv. '4-season leaf'

Cross combination	PGI ¹⁾	No. of flowers pollinated	No. of ovaries cultured	No. of embryos cultured	No. of seeds obtained	No. of hybrids (generation)
Amphidiploid self	1.8	110	100	0	—	—
Amphidiploid × <i>R. sativus</i>	2.8	110	100	6	—	2 (BC ₁)
<i>R. sativus</i> × amphidiploid	2.4	110	100	0	—	—
Amphidiploid × <i>D. tenuifolia</i>	2.8	60	50	0	—	—
<i>D. tenuifolia</i> × amphidiploid	3.8	100	90	4	—	1 (BC ₁)
BC ₁ × <i>R. sativus</i>	4.0	870	—	—	165	102 (BC ₂)
<i>R. sativus</i> × BC ₁	3.2	58	—	—	15	12 (BC ₂)

¹⁾ Pollen Germination Index according to Matsuzawa (1983), 0 ≤ PGI ≤ 4.

Table 4. Distribution of somatic chromosome numbers in BC₂ hybrids derived from reciprocal crossings between BC₁ hybrids (2n=29, DtRR) and *R. sativus* cv. '4-season leaf' (2n=18, RR)

Hybrids	Cross combination in BC ₁	No. of plants observed	Somatic chromosome number (2n)					
			18	19	20	21	22	23
BC ₂ hybrid	BC ₁ × RR	89	49	22	14	3	0	1
		(%)	55.1	24.7	15.7	3.4	0.0	1.1
BC ₂ hybrid	RR × BC ₁	12	2	5	2	0	2	1
		(%)	16.7	41.6	16.7	0.0	16.7	8.3

Fig. 1. Comparison of morphological characters of parental plants, an amphidiploid, two BC₁ hybrids and four BC₂ hybrids. (A) *D. tenuifolia*, (B) *R. sativus* cv. '4-season leaf', (C) amphidiploid (DtDtRR), (D) BC₁ hybrid (DtRR), (E) BC₁ hybrid (DtDtR), (F)~(I) BC₂ hybrids are *D. tenuifolia* monosomic chromosome addition lines of *R. sativus* (MALs).

Fig. 2. Meiotic and mitotic chromosomes of an amphidiploid (DtDtRR), two BC₁ hybrids (DtRR and DtDtR) and a BC₂ hybrid (MALs). (A) PMC of an amphidiploid (DtDtRR, 2n=40) forming a 20 to 20 chromosome segregation at metaphase II, (B) PMC of a BC₁ hybrid (DtRR, 2n=29) forming 9II+11I at meiotic metaphase I, (C) PMC of a BC₁ hybrid (DtDtR, 2n=31) forming 11II+9I at meiotic metaphase I, (D) Root tip cell of a BC₂ hybrid with 2n=19 (MALs).

The chromosome configuration type of 11II + 9I at metaphase I in a BC₁ hybrid (2n=31) derived from the crossing of *D. tenuifolia* × amphidiploid suggested the presence of two *D. tenuifolia* genomes (DtDt, 2n=22) and one *R. sativus* genome (R, n=9) (Fig. 2C). Pollen fertility of the BC₁ hybrids with DtRR and DtDtR genomes was 48.6% and 14.8%, respectively.

Production of BC₂ hybrids and their morphological and cytological characteristics

Successive backcrossings of two BC₁ hybrids (2n=29, DtRR) to *R. sativus* and reciprocal crossing were performed to produce BC₂ hybrids (Table 3). In the crossing of two BC₁ hybrids × *R. sativus*, the pollen tubes of *R. sativus* elongated regularly near the ovules of the BC₁ hybrid, accounting for a mean PGI of 4.0. One hundred and sixty-five seeds were obtained from 870 flowers pollinated without embryo rescue, from which 102 BC₂ hybrids carrying *D. tenuifolia* cytoplasm were grown. In the reciprocal crossing of *R. sativus* × BC₁ hybrid, the pollen tubes of the BC₁ hybrid elongated vigorously near the ovules of *R. sativus* (PGI, 3.2). Fifteen seeds were harvested from 58 flowers pollinated by conventional pollination, from which 12 reciprocal BC₂ hybrids carrying the *R. sativus* cytoplasm were obtained.

The somatic chromosome numbers of 89 BC₂ and 12

reciprocal BC₂ hybrids ranged from 2n=18 to 2n=23 and no plants with more than 2n=24 were generated (Table 4). The BC₂ hybrids with 2n=18 which were considered to correspond to the alloplasmic *R. sativus* carrying the *D. tenuifolia* cytoplasm were obtained in the highest frequency, followed by 2n=19 (Fig. 2D), 2n=20, 2n=21 and 2n=23. The reciprocal BC₂ hybrids with 2n=19 were obtained in the highest frequency (41.6%), followed by 2n=18, 20 and 22 and 2n=23. Compared with *R. sativus* cv. '4-season leaf', all the hybrids with 2n=19 exhibited distinctive and diagnostic features based on the morphological characters, depending upon the presence of an added chromosome of *D. tenuifolia* genome (Fig. 1F~1I).

Discussion

Wide hybridization in Brassicaceae has been carried out for the introgression of favorable agronomic traits from wild species into cultivated crops. Hybrid progenies produced by wide hybridization were classified into the following five lines: synthetic amphidiploid line, alien gene(s) introgression line, alloplasmic line, monosomic alien chromosome addition line and monosomic alien chromosome substitution line (Matsuzawa *et al.* 1996). These genetically modified progenies may be able to investigate the genetic effects on valuable agronomic traits at the genomic, chromosomal and genic levels, and estimate the performance of genetic improvement in cultivated crops. In this study, we produced hybrids with various genome constitutions (DtDtRR, DtRR and DtDtR genomes), *D. tenuifolia* MALs of auto- and alloplasmic *R. sativus* (MALs) and alloplasmic *R. sativus* (RR) via intergeneric F₁, BC₁ and BC₂ hybrids between *D. tenuifolia* (C₃-C₄ intermediate species, DtDt) and *R. sativus* (C₃ species, RR).

In the intergeneric reciprocal crossings between *D. tenuifolia* and the five cultivars of *R. sativus*, pre-fertilization barriers operated in the crossing of *R. sativus* × *D. tenuifolia*. However, the pre-fertilization barriers were less strong when the *R. sativus* cv. 'Aokubi-Miyashige-Nagabuto' and '4-season leaf' were used as pollen parents (Table 1). It appeared that the pre-fertilization barriers largely depended upon the crossing combination and direction. Such unilateral incompatibility had been reported in other cases of wide hybridization of *Brassica* and *Raphanus* crops with wild species (Nanda Kumar *et al.* 1988, Batra *et al.* 1990, Gundimeda *et al.* 1992, Bang *et al.* 1996a, 1996b, 1997a). On the other hand, in the reciprocal crossings between the induced amphidiploid and its parental plants, namely *D. tenuifolia* and *R. sativus*, the pre-fertilization barriers were less strong than those in the reciprocal crossings between *D. tenuifolia* and *R. sativus*, although the amphidiploid showed a higher degree of self-incompatibility. Bang *et al.* (1996a) also reported that in the amphidiploid between *M. arvensis* and *R. sativus* the pre-fertilization barriers were hardly expressed when backcrossing to *R. sativus* was conducted, where the BC₁ hybrids could be obtained

through ovary culture followed by embryo culture. In the present study, the post-fertilization barriers in the crossing processes of F₁ and BC₁ hybrids could be overcome by the use of embryo rescue. Accordingly, the polyploidization of the F₁ hybrids obtained from wide hybridization in Brassicaceae could not only overcome the pre-fertilization barriers but also promote both pollen fertility and seed setting. The use of ovary culture followed by embryo culture may be able to overcome the post-fertilization barriers in wide hybridization and backcrossing of F₁ hybrids to parent species.

The BC₁ hybrids (DtRR) exhibited the chromosome configuration type of 9II+1II at metaphase I of PMCs. This chromosome configuration indicated that the BC₁ hybrids had two R genomes and one Dt genome, and that heterogenetic pairing between the two genomes did not occur. Namai (1987) pointed out that heterogenetic pairing might be a prerequisite to introgress useful traits into another species concerned. On the other hand, he also postulated that the structural and qualitative changes of chromosomes (translocation, inversion, deficiency, elimination etc.) caused by heterogenetic pairing may not be suitable for the development of a complete series of MALs. In the present study, many BC₂ hybrids with 2n=18~23 were generated through both female and male gametes of the BC₁ hybrids, and 24.7 % of the BC₂ hybrids and 41.6% of the reciprocal BC₂ hybrids were assumed to be MALs based on the observation of somatic chromosome numbers. Kaneko *et al.* (2001) reported that the proportions of the *R. sativus*-*B. rapa* MALs generated in the progenies through female and male gametes of the BC₁ hybrids (RRA genome) were 50.9% and 40.0%, respectively, although in the BC₁ hybrids (RRA genome) more heterogenetic pairings were observed than in the present BC₁ hybrids (DtRR genome). As for the development of MALs with the genetic background of *R. sativus*, the proportions of MALs produced through female gametes of the BC₁ hybrids were 25% for *B. oleracea* (Kaneko *et al.* 1987) and 20% for *M. arvensis* (Bang *et al.* 2002).

Matsuzawa *et al.* (1996) classified the five lines with genetic modifications into autoplasmic and alloplasmic ones. These lines are suitable materials for estimating the effect of the cytoplasm on morphological and other traits. Hybrids carrying the *R. sativus* cytoplasm (autoplasmic line) could not be produced in this study due to the unilateral incompatibility in the intergeneric crossing of *R. sativus* × *D. tenuifolia*. However, some autoplasmic MALs were produced from the five reciprocal BC₂ hybrids (2n=19) that were generated through male gametes of the BC₁ hybrids (DtRR genome). Bang *et al.* (1996a) reported similar instances in the reciprocal crossings between *M. arvensis* and *R. sativus*, where the F₁ hybrids could not be obtained when *R. sativus* was used as pistillate parent. However, seven *M. arvensis* autoplasmic MALs of *R. sativus* could be produced through male gametes of eight *M. arvensis* alloplasmic MALs of *R. sativus* (Bang *et al.* 1997b, 2002). Therefore, if eleven *D. tenuifolia* alloplasmic MALs of *R. sativus* were produced from the BC₂ hybrids obtained in this study and

then if they displayed pollen fertility, complete sets of eleven *D. tenuifolia* autoplasmic MALs of *R. sativus* might be obtained through male gametes of the alloplasmic MALs in addition to the BC₁ hybrids. The novel intergeneric hybrids produced in this study could become reliable and potential materials to investigate the genetic effects on C₃-C₄ intermediate traits at genomic, chromosomal and genic levels, and to estimate the performance of genetic improvement in Brassicaceae.

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