

Cytogenetical Stability and Fertility of an Intergeneric Amphidiploid Line synthesized from *Brassica maurorum* Durieu. and *Raphanus sativus* L.

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Résumé

An intergeneric amphidiploid line, *Brassicoraphanus* ($2n=34$, FFRR), which was synthesized from the cross of *Brassica maurorum* Durieu. ($2n=16$, FF) \times *Raphanus sativus* L. (cv. "4-season leaf", $2n=18$, RR), showed well-regulated chromosome behavior at the meiosis of pollen mother cells (PMCs), which resulted in higher levels of both pollen and seed fertility. All *Brassicoraphanus* plants obtained by selfing through three generations (S_1F_1 to S_3F_1) were true amphidiploids with the sum of the somatic chromosome number of parents being $2n=34$. They were intermediate between their parents in several morphological characteristics, such as leaf shape, inflorescence and flower characters except for white petals and the absence of root thickening. The BC_1 plants obtained from the backcrossing of the S_2F_1 plants with *B. maurorum* were sesquidiploid ($2n=25$), exhibiting the putative chromosome number with the genomic constitution of FFR. In the present study, the amphidiploid and sesquidiploid plants produced by selfing or sib cross and backcross, respectively, from the *Brassicoraphanus* will be used as bridge plants for the breeding of the hybrid progenies advocated by MATSUZAWA et al. (1996).

Introduction

In the cultivated *Brassica* crops, there are amphidiploid species, known as *Brassica juncea* (L) Czern. ($2n=36$, AABB), *B. napus* L. ($2n=38$, AACC) and *B. carinata* Braun. ($2n=34$, BBCC) which have been naturally synthesized from the three diploid *Brassica* species of *B. campestris* L. ($2n=20$, AA), *B. nigra* Koch. ($2n=16$, BB) and *B. oleracea* L. ($2n=18$, CC) (U, 1935). A number of interspecific and intergeneric amphidiploid hybrids, including the natural amphidiploid species described above, have been artificially synthesized by sexual and somatic hybridization between *Brassica* crops and wide relatives. Amphidiploid hybrids potential as new crop species and as a provider of new material for breeding and genetical work has been evaluated (NAMAI, 1987; MATSUZAWA et al., 1996). However, because of the lower fertility encountered in their breeding process, only a few are now available for critical research.

Recently, the amphidiploid hybrids are being used as the bridge plant to breed the following five lines: I. Synthetic amphidiploid line (SADL), II. Alien gene(s) introgression line (AGIL), III. Alloplasmic line (ALPL), IV. Monosomic alien chromosome addition line (MAAL) and V. Monosomic alien chromosome substitution line (MASL) (MATSUZAWA et al., 1996). A number of these lines have been produced from the intergeneric cross between *R. sativus* and wild allied genera (BANG et al., 1997a; KANADA and KATO, 1997; WATANABE et al., 1997): SADLs between *R. sativus* and *Brassica maurorum*, *B. fruticulosa* and *B. oxyrrhina*, ALPLs between *R. sativus* and *B. oxyrrhina*, *Eruca vesicaria*, *Moricandia arvensis*, *Sinapis arvensis* and *S. turgida* and MAALs between *R. sativus* and *E. sativa*, *M. arvensis* and *S. arvensis*. Cytogenetical stability and higher fertility of the

bridge plant may be a prerequisite for the development of these five lines. MATSUZAWA et al. (1997) investigated the cytogenetical stability and fertility of a new *Brassicoraphanus* synthesized from *B. oxyrrhina* and *R. sativus*, and suggested that the new *Brassicoraphanus* was true-breeding line with a higher and less variable fertility than any bridge plants developed previously.

In this study, our goal was to inspect the cytogenetical stability and fertility of a new *Brassicoraphanus*, synthesized from *Brassica maurorum* and *R. sativus*, which will be used to produce the five hybrid lines described above. The research has three objectives: (1) to investigate the chromosome behavior at meiosis of pollen mother cells (PMCs) in successive generations of the amphidiploid line; (2) to investigate the pollen fertility and seed setting of the amphidiploid line produced by selfing and sib cross; and (3) to produce the BC₁ plants from the amphidiploid line by backcross with the parental species.

Materials and Methods

The amphidiploid plants, *Brassicoraphanus* ($2n=34$, FFRR), were developed by doubling the chromosomes of intergeneric F₁ hybrids which were synthesized by applying ovary culture followed by embryo culture from the intergeneric cross between *Brassica maurorum* Durieu. ($2n=16$, FF) and *Raphanus sativus* L. (cv. "4-season leaf", $2n=18$, RR) (BANG et al., 1997b). The seeds of *B. maurorum* were supplied by the Laboratory of Plant Breeding, Tohoku University, Sendai, Japan, from Cruciferae genertic stocks. *R. sativus* cv. "4-season leaf" is one of the assessions in the Laboratory of Plant Breeding, Utsunomiya University, Utsunomiya, Japan.

Young buds were emasculated a day before anthesis, immediately pollinated with fresh pollens and then bagged for a week. Two F₁ amphidiploid plants were selfed by hand-pollination to generate the next generation (S₁F₁). The successive generations to the S₃F₁ were also obtained by self-pollination. The seed setting in the self-pollination of the amphidiploid plants from the S₁F₁ to the S₃F₁ generation was compared with that of the sib cross in the S₂F₁ and S₃F₁ generation. To produce BC₁ plants, three S₂F₁ and six S₃F₁ amphidiploid plants were backcrossed with *B. maurorum* and *R. sativus*. All of the amphidiploid plants were grown in 18 cm diameter unglazed pots in a greenhouse, and then identified on the basis of their morphology and mitotic chromosome counting.

Mitotic chromosomes in root-tip cells were counted using the Feulgen stain squash method followed by a 1% acetocarmine staining. Meiotic chromosome behavior was examined in PMCs using the 1% acetic orcein smear method. Pollen tetrads were counted after a 1% acetic orcein staining. Pollen fertility was ascertained by observing one thousand pollen grains after staining with a 1% acetocarmine. The pollen grains taking up the stain and having a round shape were considered to be fertile.

Results

Fertility and morphology of the amphidiploid from the F₁ to the S₃F₁ generations

Pollen fertility and seed setting obtained through selfing, sib cross and backcross of the amphidiploid plants, *Brassicoraphanus*, from the F₁ to the S₃F₁ generations are shown in Table 1. In S₁F₁, S₂F₁ and S₃F₁ generations, the pollen fertility of amphidiploid plants was approximately 90% (Fig. 1A), and they exhibited more stable and less variable pollen fertility in the subsequent S₃F₁ generation, ranging from 78.8% to 97.2%. On the other hand, the seed setting obtained through selfing decreased as the generation progressed. The highest seed setting (192.3%) was observed in

Table 1. Fertility of the amphidiploid *Brassicoraphanus* ($2n=34$) from the F_1 to the S_3F_1 generations in selfing, sibcross and backcross with the parental species of *B. maurorum* and *R. sativus*

Generation	No. of plants	Pollen fertility (%)	Seed setting (%)			
			Selfing	Sib cross	Backcross	
					<i>B. maurorum</i>	<i>R. sativus</i>
F_1	2	81.6 ²⁾ (75.5–87.6)	53.3 (47.2–59.3) ⁴⁾	n.d. ³⁾	n.d.	n.d.
S_1F_1	18	90.9 (56.1–98.6)	99.5 (34.4–192.3)	n.d.	n.d.	n.d.
S_2F_1	3	87.9 (64.4–95.2)	37.8 (2.9–87.0)	22.1 (7.4–35.0)	204.5 (5.0–544.2)	0
S_3F_1	6	90.2 (78.8–97.2)	29.5 (0–66.7)	48.1 (0–177.3)	217.0 (0–368.8)	0

¹⁾ Seed setting (%) = (No. of seeds obtained / No. of flowers pollinated) × 100

²⁾ This data, the pollen fertility of F_1 , was previously published by BANG et al. (1997b).

³⁾ n.d.; Not determined

⁴⁾ (Range)

the S_1F_1 generation in which the mean seed setting was 99.5 %. In the S_3F_1 generation, the seed setting was lower, with the mean of 29.5 % ranging from 0 to 66.7 %. When S_3F_1 plants were sib crossed, the mean seed setting percentage was 48.1 % and the seeds were obtained from the pods developed at the upper site of each inflorescences (Fig. 1B). In the backcross of the S_2F_1 and S_3F_1 plants with *B. maurorum*, the plants showed higher seed settings than that of selfing and sib cross and their mean values were 204.5 % and 217.0 %, respectively. When the S_2F_1 and S_3F_1 plants were backcrossed with *R. sativus*, however, they failed in seed setting.

All the amphidiploid plants (F_1 to S_3F_1) were intermediate in several morphological characteristics between their parents, such as leaf shape, inflorescence and flower, except for white petals as in the case of *R. sativus* and the absence of root thickening as in the case of the *B. maurorum* (Fig. 1C, D and E). In the selfing, sid cross and backcross, more uniform and fertile seeds were obtained from each amphidiploid plant throughout the successive generations (Fig. 1F). A thousand seed-weight of amphidiploid and sesquidiploid lines resulting from in the selfing, sid cross and backcrosses had ca. 2.0 g, 1.8 g and 0.5 g, respectively, whereas that of their original parents, *B. maurorum* and *R. sativus* cv. "4-season leaf" were 0.2 g and 19.5 g, respectively.

Cytogenetical stability of the amphidiploid lines

The plants obtained from each amphidiploid plant in several generations by selfing were all true amphidiploid hybrids with the sum of the somatic chromosome number of their parents species being $2n=34$ (Table 2, Fig. 1G). The BC_1 plants obtained from the backcross of the S_2F_1 plants with *B. maurorum* had $2n=25$, showing the putative chromosome number with the genomic constitution of FFR (Table 2).

Chromosome behaviors at meiosis of PMCs were examined in the amphidiploid plants from the S_1F_1 to the S_3F_1 generations (Table 3). They showed relatively regular features in PMCs forming 17 bivalents and nuclear plates with 17 chromosomes at metaphase I and II (MI and MII), respectively (Fig. 1H and I). In these amphidiploid plants of S_1F_1 , S_2F_1 and S_3F_1 , the mean

chromosome association types at M I were $16.8_n + 0.4_i$, $16.9_n + 0.2I$ and $16.8_n + 0.3_i$, respectively and the chromosome number of nuclear plates at M II ranged from 16 to 18. The frequency of pollen tetrad increased steadily with the progression of the generation. In the S_3F_1 generation, the frequency of pollen tetrad was 99.3 %, ranging from 99.1 % to 100 %.

Table 2. Somatic chromosome numbers of amphidiploid plants ($2n=34$, FFRR) in successive generations and BC_1 plants ($2n=25$, FFR) obtained from amphidiploid by backcross with *B. maurorum* ($2n=16$, FF)

Generation	No. of plants observed	Chromosome number in root tip cells ($2n$)						
		≤ 24	25	26	~	33	34	$35 \leq$
S_1F_1	15							15
S_2F_1	14							14
S_3F_1	10							10
BC_1 ¹⁾	6				6			

1) BC_1 plants were obtained from S_2F_1 amphidiploid by backcross with *B. maurorum*

Table 3. Chromosome behavior at meiosis of PMCs of the amphidiploid *Brassicoraphanus* ($2n=34$, FFRR) in S_1F_1 , S_2F_1 and S_3F_1 generation

Generation	No. of plants	Chromosome associations at M I			Chromosome number of nuclear plate at M II					Frequency of pollen tetrad (%)	
		No. of PMCs observed	II	I	No. of N.P.s ¹⁾ observed	16	16½	17	17½		18
S_1F_1	14	700	16.8 (14-17) ²⁾	0.4 (0-6)	1076	74	19	921	20	42	94.6 (86.6-100)
S_2F_1	6	150	16.9 (16-17)	0.2 (0-2)	300			300			95.4 (93.2-98.8)
S_3F_1	9	180	16.8 (16-17)	0.3 (0-2)	360	2		356		2	99.3 (99.1-100)

¹⁾ N. Ps; Nuclear plates

²⁾ (Range)

Discussion

One of the barriers encountered in crop improvement, using a wide hybridization with remotely related species, is the hybrid sterility resulting from the lack of homologous pairing of chromosomes. Such sterility may be overcome by mitotic chromosome doubling. In intergeneric F_1 hybrids synthesized from *R. sativus* and allied genera, such as *Sinapis*, *Moricandia* and *Brassica*, BANG et al. (1996a,b, 1997b) observed that the amphidiploid hybrid exhibited a more stable chromosome behavior at meiosis of PMCs and a higher pollen fertility than the amphihaploid one. Within the five lines (SADL, AGIL, ALPL, MAAL and MASL) advocated by MATSUZAWA et al. (1996), a number of ALPLs and MAALs have been produced from the intergeneric amphidiploid hybrids between *R. sativus* and wild allied genera (BANG et al., 1995, 1997c; WATANABE et al., 1997; KANADA and KATO, 1997). For the production of these five lines, therefore, it may be also be a prerequisite that the self-, sib cross- and backcross progenies generated from the amphidiploid hybrids are viable and fertile.

MATSUZAWA et al. (1997) investigated whether an amphidiploid line synthesized from *B. oxyrrhina* and *R. sativus* could be used as bridge plant to produce the five lines, and then offered a new *Brassicoraphanus* which was true-breeding amphidiploid line with favorable fertility. This investigation resulted in the development of the new amphidiploid, *Brassicoraphanus*, which is a true-breeding amphidiploid line with favorable fertility and can be used as a bridge plant with great potential to produce the five lines.

The new *Brassicoraphanus* exhibited a well-regulated chromosome behavior at meiosis of PMCs and a higher pollen fertility with the progression of the generation (Table 1 and 3, Fig. 1A, H and I). The seed fertility of the present amphidiploid was lower in selfing with the progression of the generation, whereas the seed fertility in sib cross was higher (Table 1). Moreover, many seeds were harvested from the pods developed on the upper site of the inflorescence (Fig. 1B). Based on these results, it might be suggested that the self-incompatibility system and/or inbreeding depression may operate in this new *Brassicoraphanus*, although the expression of self-incompatibility is inferior to the younger buds with the female fertility.

It was reported that the seed fertility in *Brassicoraphanus* and *Raphanobrassica* synthesized from *Brassica* and *Raphanus* was lower than the parents because of their meiotic irregularity and their cross incompatibility under the genetic control (McNAUGHTON, 1973; TOKUMASU and KATO, 1980; IWASA and ELLERSTROM, 1981; DOLSTRA, 1982; KATO and TOKUMASU, 1983; MATSUZAWA et al., 1985). The *Brassicoraphanus* line, which was synthesized from *B. oxyrrhina* and *R. sativus* by MATSUZAWA et al. (1997) showed regular chromosome behavior at meiosis of PMCs in early generations, resulting in higher seed fertility of 4.2 seeds per pod. The new *Brassicoraphanus* developed approximately 2 seeds per pollinated flower in some S₁F₁ plant when the self-pollination was performed (Table 1). Moreover, the new *Brassicoraphanus* produced approximately 5 seeds per flower in backcrossing with *B. maurorum* at the S₂F₁ generation. These results may suggest that the new *Brassicoraphanus* has more than 5 fertile ovules per flower. Accordingly, if some incompatible factors described above could be favorably eliminated, the new *Brassicoraphanus* may be able to produce more than 5 seeds per flower in both self and sib cross.

In the backcross of the *Brassicoraphanus* with the parental species of *B. maurorum* and *R. sativus*, the former showed higher seed fertility, whereas the latter failed in seed setting (Table 1). In the intergeneric cross of *B. maurorum* × *R. sativus*, hybrid embryos could be obtained only in ovary culture because of the post-fertilization barriers (BANG et al., 1997b). If the present *Brassicoraphanus* has the same crossability system inherited from *B. maurorum*, the hybrid plants of *Brassicoraphanus* × *R. sativus* may be produced by using the embryo rescue method. At any rate, the characteristics of crossability in the cross of *Brassicoraphanus* × *R. sativus* may be elucidated by the analyses of the pollen germination and the development of embryo *in vivo* and *in vitro* previously performed by BANG et al. (1997b).

The amphidiploid and sesquidiploid plants obtained by selfing, sib cross and backcross of the *Brassicoraphanus* (*B. maurorum* × *R. sativus* amphidiploid line) in the present study will be used as bridge plants for the breeding of the hybrid progenies designated by MATSUZAWA et al. (1996).

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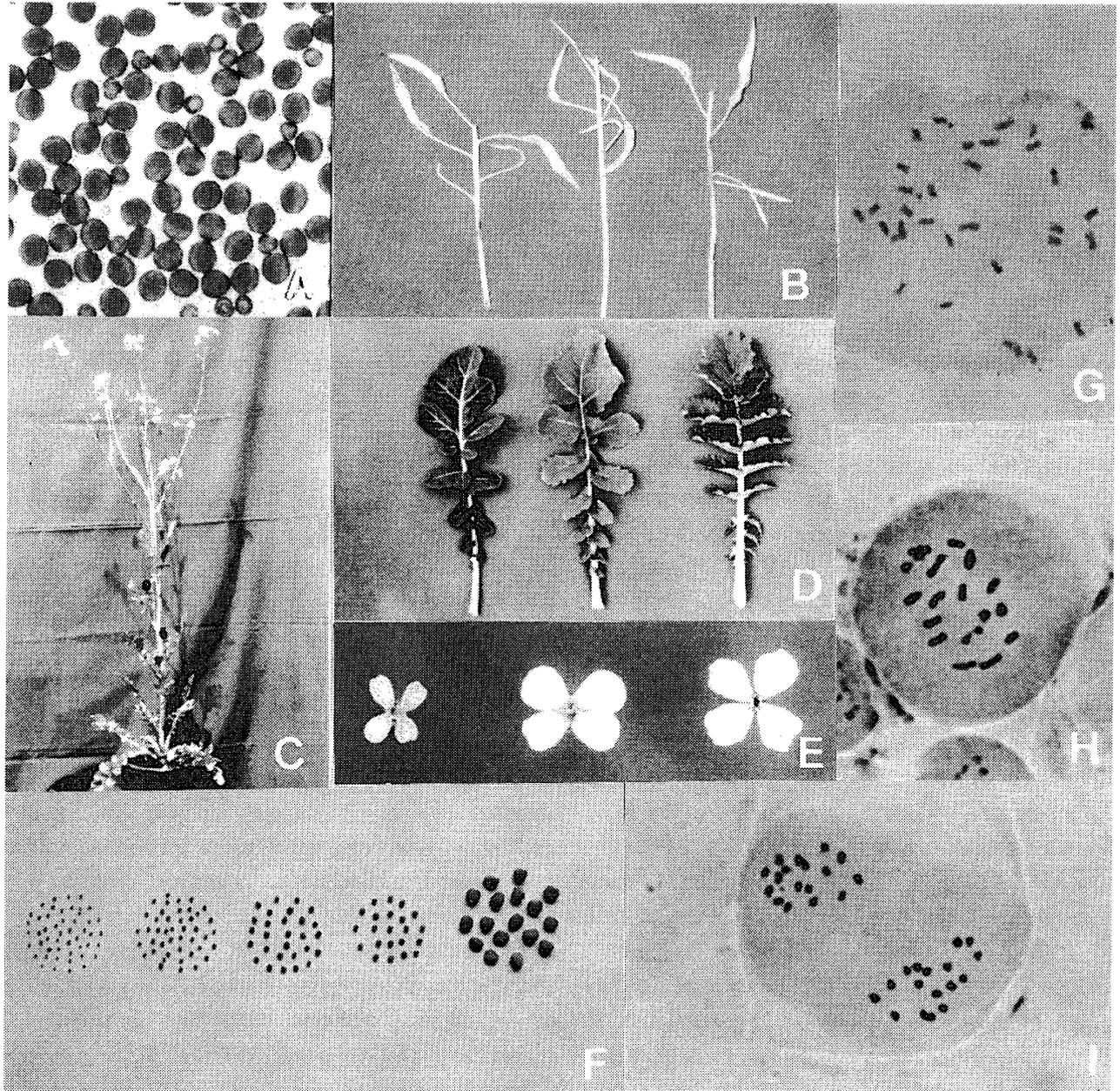


Fig. 1 Morphological and cytological characteristics of the *Brassicoraphanus* ($2n=34$, FFRR), *B. maurorum* ($2n=16$, FF) \times *R. sativus* ($2n=18$, RR).

A) Fertile pollen grains which were stained with 1% acetocarmine.

B) Fertile pods predominantly developed at the upper site of inflorescence by sib cross.

C) Plant type of a *Brassicoraphanus* in the flowering stage.

D) leaves, left : *B. maurorum*; middle : *Brassicoraphanus* and right : *R. sativus* cv. "4-season leaf".

E) Flowers, left : *B. maurorum*; middle : *Brassicoraphanus* and right : *R. sativus* cv. "4-season leaf".

F) Seeds: *B. maurorum*; sesquidiploid, *Brassicoraphanus* (sib and self), and *R. sativus* cv. "4-season leaf" (left to right).

G) Somatic chromosomes ($2n=34$) in a root tip.

H) PMC with 17_n chromosome association type at M I.

I) PMC with (17-17) distribution type at M II.

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Brassica maurorum Durieu. と *Raphanus sativus* L. との人為合成複二倍体植物の
細胞遺伝学的安定性と稔性

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摘 要

Brassica maurorum ($2n=16$, FFゲノム) とダイコン(*Raphanus sativus*, $2n=18$, RRゲノム) との人為合成複二倍体植物 *Brassicaraphanus* ($2n=34$, FFRRゲノム) は、世代が進むとともに安定した染色体行動と高い花粉稔性を示し、比較的高い結実を示した (Table 1と3)。また、*Brassicaraphanus* の自殖によって得られた後代植物 ($S_1F_1 \sim S_3F_1$) はいずれも $2n=34$ の複二倍体であり (Table 2)、形態的にも両親種の間を示した (Fig. 1C, DおよびE)。*Brassicoraphanus* を種子親に育成親 (*B. maurorum* とダイコン) を花粉親に用いた戻交雑において、*Brassicaraphanus* × *B. maurorum* の組合せは高い結実を示したが、い

い結実を示したが、*Brassicaraphanus* × ダイコンでは不稔であった (Table 1)。このうち、 S_2F_1 世代の *Brassicaraphanus* × *B. maurorum* の組合せから得た BC_1 植物は、 $2n=25$ の染色体数をもつ二基三倍体 (FFRゲノム) であった。

本研究で新しく育成した複二倍体種と二基三倍体種は、MATSUZAWA et al. (1996) が提唱した I. 人為合成複二倍体、II. 異種遺伝子導入系統、III. 異種細胞質系統、IV. 異種染色体添加系統および V. 染色体置換系統を育成するための橋渡し植物として利用できるものと思われた。

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