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Development of synthetic *Brassica* amphidiploids by reciprocal hybridization and comparison to natural amphidiploids

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Abstract. In a previous study we proposed that cytoplasmic genomes have played an important role in the evolution of *Brassica* amphidiploid species. Based on this and other studies, we hypothesized that interactions between the maternal cytoplasmic genomes and the paternal nuclear genome may cause alterations in genome structure and/or gene expression of a newly synthesized amphidiploid, which may play an important role in the evolution of natural amphidiploid species. To test this hypothesis, a series of synthetic amphidiploids, including all three analogs of the natural amphidiploids *B. napus*, *B. juncea*, and *B. carinata* and their reciprocal forms, were developed. These synthetic amphidiploids were characterized for morphological traits, chromosome number, and RFLPs revealed by chloroplast, mitochondrial, and nuclear DNA clones. The maternal transmission of chloroplast and mitochondrial genomes was observed in all of the F_1 hybrids examined except one hybrid plant derived from the *B. rapa* × *B. oleracea* combination, which showed a biparental transmission of organelles. However, the paternal chloroplast and mitochondrial genomes were not observed in the F_2 progeny. Nuclear genomes of synthetic amphidiploids had combined RFLP patterns of their parental species for all of the nuclear DNA clones examined. A variation in fertility was observed among self-pollinated progenies of single amphidiploids that had completely homozygous genome constitutions. Comparisons between natural and synthetic amphidiploids based on restriction fragment length polymorphism (RFLP) patterns indicated that natural amphidiploids are considerably more distant from the progenitor diploid species than the synthetic amphidiploids. The utility of these synthetic amphidiploids for investigating the evolution of amphidiploidy is discussed.

Key words: *Brassica* – Interspecific hybridization – Amphidiploid – RFLP – Evolution – Nuclear – cytoplasmic interaction

Introduction

One of the most important questions on the evolution of polyploidy in plants regards the mechanisms of stabilization of newly formed polyploids. How fast does this process occur and what factors have been involved? Previous studies have indicated that the stabilization of polyploids involves: (1) chromosome diploidization in which the homoeologous pairing of chromosomes is suppressed, and thus the chromosome pairing of polyploids resembles that of diploids, and (2) genetic diploidization in which duplicate genes are either silenced or expressed at reduced levels. A well-known example of diploidization at the chromosome level has been described for hexaploid wheat in which a dominant gene, termed *Ph*, was found to be responsible for the suppression of homoeologous pairing (Riley and Chapman 1958; Sears 1976). However, *Ph*-like genes have not been found in other polyploids, and genetic diploidization in these species appears to be more complex and controversial. Wilson et al. (1983) reported the loss of duplicate gene expression in tetraploid *Chenopodium*, and Gastony (1991) found gene silencing in a polyploid homosporous fern. In other

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cases, duplicate genes originating from diploid progenitors were both expressed in the polyploids (Demaggio and Lambrukos 1974; Roose and Gottlieb 1976; Coulthart and Denford 1982; Chen et al. 1989).

Amphidiploid species are a form of polyploids that have evolved from interspecific hybridization between two or more diploid species, either through the fusion of unreduced gametes or through interspecific hybridization followed by spontaneous chromosome doubling. Since in most cases cytoplasmic genomes are maternally inherited, amphidiploid species possess distinct nuclear genomes housed in the maternal cytoplasm. In several plant species, foreign combinations of nuclei and cytoplasms cause the disruption of normal cellular function, resulting in male sterility (Edwardson 1970; Erickson et al. 1986; Escote-Carlson et al. 1982; Pearson 1981) or chlorosis (Shiga 1980; Kata and Tokumasu 1978). There is evidence that the introduction of components from foreign nuclear genomes can influence changes in organelle genomes, especially in mitochondrial genomes (Escote-Carlson et al. 1990; MacKenzie et al. 1988). Cytoplasmic genomes also may influence changes in nuclear genomes, especially when foreign cytoplasms and nuclei are combined, such as in amphidiploid species.

In a previous phylogenetic study based on nuclear restriction fragment length polymorphisms (RFLPs), we found that when the parental diploid species of *Brassica* amphidiploids had highly differentiated cytoplasms, the nuclear genomes of amphidiploids originating from the male donors were altered considerably more than the nuclear genomes originating from the female parents (Song et al. 1988). This observation led us to hypothesize that interactions between nuclear and cytoplasmic genomes may cause modifications in portions of the paternal nuclear genome and/or the maternal cytoplasmic genomes, which might play an important role in the stabilization of newly synthesized amphidiploids during subsequent sexual cycles. Amphidiploid *Brassica* species are an excellent system for testing this hypothesis. First, the genome relationships between the three naturally occurring *Brassica* amphidiploids, *B. juncea* (n = 18), *B. napus* (n = 19), and *B. carinata* (n = 17) and their progenitor diploid species, *B. rapa* (n = 10), *B. nigra* (n = 8), and *B. oleracea* (n = 9), have been well-established based on evidence from interspecific hybridization, cytogenetics, isozymes, and nuclear RFLPs (U 1935; Prakash and Hinata 1980; Coulthart and Denford 1982; Song et al. 1988). Also, the cytoplasmic origins of these *Brassica* amphidiploids have been determined by restriction fragment analysis of chloroplast DNA (Erickson et al. 1983; Palmer et al. 1983). Finally, in vitro culture techniques have been developed (Bajaj et al. 1986), making it possible to obtain a series of artificially synthesized amphidiploids of *Brassica*.

Our main goal is to determine if nuclear genomes of amphidiploid *Brassica* species undergo substantial short-term changes after synthesis from diploid progenitors and if cytoplasmic factors influence the direction of nuclear genome change and/or the patterns of gene expression in synthetic amphidiploids. To test our hypothesis, the natural amphidiploid species cannot be used as research material because of our inability to know the exact diploid progenitors. It is crucial to create a series of reciprocal amphidiploids in which the same sets of nuclear genomes are harbored in different cytoplasms. Artificially synthesized amphidiploids would provide a well-controlled experimental system that could be monitored for potential genome modification at the molecular level. In this paper, we report on a series of artificial amphidiploids that were synthesized by reciprocal interspecific crosses followed by embryo rescue and chromosome doubling. These reciprocal amphidiploids have been characterized by chromosome counting and by nuclear DNA, chloroplast DNA (ctDNA) and mitochondrial DNA (mtDNA) RFLPs. Furthermore, synthetic and natural amphidiploid species were compared for RFLP patterns of both the cytoplasmic and nuclear genomes.

Materials and methods

Plant materials

Five *Brassica* diploid accessions, 2 from *B. rapa* (A genome), 2 from *B. nigra* (B genome), and 1 from *B. oleracea* (C genome) were used in reciprocal interspecific crosses. For comparison between natural and synthetic amphidiploids, these accessions plus 1 accession from *B. oleracea*, 3 accessions of *B. nigra*, 6 accessions of *B. carinata*, 18 accessions of *B. napus*, and 17 accessions of *B. juncea* were analyzed for ctDNA, mtDNA and nuclear DNA RFLPs (Table 1).

Hybridization

Single plants were used as parents in crosses so that the genotypes of hybrids could be traced to specific parent plants. Pollinations were made 2 days after the flowers opened, and young siliques were excised for ovary culture 6–14 days after pollination (DAP), depending on the cross combination. Three different media, W1, N1 and B5-1 (Sigma), were used for ovary culture of the different crosses in order to optimize results. For all of the cross combinations, embryos were dissected from withered ovules 1–2 weeks after ovary culture and transferred into MS medium (Sigma) plus 800 mg/l glutamine, 500 mg/l casein hydrolysate, and 50 g/l sucrose. Young seedlings were transplanted into 10 cm × 10 cm square pots containing Jiffy Mix (Jiffy Products of America, Inc., W. Chicago, Ill.) and then transplanted into 25-cm diameter pots about 1 month later.

Chromosome doubling

The hybridity of F_1 plants was confirmed on the basis of morphology and chromosome number, and cuttings were subsequently made from true hybrids. The cuttings were dug out 10–15 days after the roots appeared, and the roots were emerged

Table 1. Accessions used for synthesis of *Brassica* amphidiploids and for RFLP analyses

Species	Accession or cultivar name (collection no.)[a]	Source[c]	Cytoplasmic type[d] ctDNA	mtDNA
B. rapa	cv Flowering Pak Choi*	Sakata	A	A
	cv Tobin*	CrGC	A	A
B. nigra	Ethiopian wild population*	CrGC	B	B
	cv WPBS*	CrGC	B	B
	Indian wild population	CrGC	B	B
	Yugoslavian wild population	CrGC	B	B
	Pakistan wild population	CrGC	B	B
B. oleracea	CrGC3-1*	CrGC	C	C
	Portuguese cabbage cv Penca de Pova	CrGC	C	C
B. carinata	cv Tex-sel	CrGC	B	B
	CrGC6	CrGC	B	B
	UCD-77-1285	CrGC	B	B
	UCD-77-1352	CrGC	B	B
	UCD-96471-490	CrGC	B	B
	Karate (4264)	WGB	B	B
	B × C synthetic (221)	UWM	B	B
	C × B synthetic (151)	UWM	C	C
B. Juncea	cv Cutlass	Canada	A	A
	cv Zhatsai	CrGC	A	A
	cv Multiple Shoots	CrGC	A	A
	cv Leafy Mustard	CrGC	A	A
	cv Southern Giant Curled	CrGC	A	A
	CrGC4	CrGC	A	A
	cv Domo	CrGC	A	A
	PI311734	NCRPIS	A	A
	PI379103	NCRPIS	A	A
	BE5-95-Agan	UPM	A	A
	PI215636	NCRPIS	A	A
	Ndakupuka (4286)	WGB	A	A
	Nan Fong (5158)	WGB	A	A
	Kai choi (5105)	UGB	A	A
	A × B synthetic (066)	UWM	A	A
	B × A synthetic (071)	UMW	B	B
B. napus	"*B. robertiana*"[b]	UPM	A	A
	Asparagus kale (6224)	WGB	A	A
	Rutabaga (3243)	WGB	M	N
	Rutabaga cv Amer. Purple Top	CrGC	M	N
	Rutabaga cv Laurentian	CrGC	M	N
	cv Altex	CrGC	M	N
	cv Regent	CrGC	M	N
	cv Reston	CrGC	M	N
	cv Brutor	France	S	S1
	cv Primor	France	M	N
	cv Major	France	M	N
	cv Bienvenue	France	M	N
	cv Hero	Canada	M	N
	cv Stellar	Canada	M	N
	cv Westar	Canada	M	N
	cv Rubin	Germany	M	N
	cv Quinta	Germany	M	N
	cv Cresor	Germany	M	N
	A × C synthetic (002)	UWM	A	A
	C × A synthetic (012)	UWM	C	C

[a] All accessions listed were included in the RFLP analyses. The accessions with * were used as parents in interspecific crosses

[b] An annual *B. napus* originally classified as *B. robertiana*

[c] UPM, Polytechnical University, Madrid, Spain; WGB, Wellsbourne Gene bank, UK; CrGC, Crucifer Genetic Corporative, University of Wisconsin, Madison, USA; NCRPIS, North Central Regional Plant Introduction Station, Ames, Iowa, USA; UWM, Dept. of Agronomy, University of Wisconsin, Madison, Wis., USA; Sakata, Sakata Seed Co., Yokohama, Japan; Canada, France, Germany, various plant breeders in these countries where the cultivars were developed

[d] Cytoplasm type determined by chloroplast DNA (ctDNA) and mitochondria DNA (mtDNA) RFLP patterns

in 0.3% colchicine for 3 h under fluorescence lights. The treated cuttings were washed thoroughly in distilled water and transplanted into Jiffy Mix without fertilization until the plants died back. After regrowth, seeds or rescued embryos of amphidiploids were obtained by self-pollinating fertile flowers.

Cytological characterization

Young leaves of amphidiploid plants were collected at 8:30 to 9:00 a.m., pretreated in a 0.002 *M* 8-hydroquinonin solution for 2.5–3.0 h, and fixed in Carnoy's solution (three parts ethanol: one

Table 2. Probes and enzymes used for detection of RFLPs

Probe[a]	Type	Plant source	Enzyme used
169	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
161	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
164	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
170	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
158	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
303	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
306	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
332	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
304	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
323	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
155	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
328	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
RG2A04	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
168	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
326	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
187	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
185	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
163	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
307	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
329	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
188	Nuclear DNA	*B. rapa* cv WR70 Days	*Eco*RI
129	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
136	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
GG4A06	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
300	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
321	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
145	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
157	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
130	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
151	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
GG5E03	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
319	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
131	Nuclear DNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI
pHA2 (a)	Ribosomal gene	Pea	*Eco*RI
pAHAZ (b)	ATPase gene	*Arabidopsis*	*Eco*RI
pHAZ (c)	Ubiquitin gene	*Arabidopsis*	*Eco*RI
pC1 (d)	Cruciferin gene	*B. napus*	*Eco*RI
pN2 (e)	Napin gene	*B. napus*	*Eco*RI
TG4C09	CtDNA	*B. rapa* cv Tobin	*Eco*RI
TG5G12	CtDNA	*B. rapa* cv Tobin	*Eco*RI
TG5G05	CtDNA	*B. rapa* cv Tobin	*Eco*RI
8a (f)	CtDNA	Orchid	*Eco*RI
19a (f)	CtDNA	Orchid	*Bam*HI
12b (f)	CtDNA	Orchid	*Bam*HI
S10.1 (g)	MtDNA	*B. rapa*	*Bam*HI
p4.4 (g)	MtDNA	*B. rapa*	*Eco*RI
S4.0 (g)	MtDNA	*B. rapa*	*Eco*RI
P5.2 (g)	MtDNA	*B. rapa*	*Eco*RI
GG4F12	MtDNA	*B. oleracea* cv Wis. Golden Acre	*Eco*RI

[a] Three digit numbers represent locus numbers for clones used to develop a *B. rapa* linkage map (Song et al. 1991). Probes preceded by RG or GG are from the same libraries as mapped probes. GG4F12 hybridizes to mtDNA (unpublished data). Probes preceded by TG are from an *Eco*RI genome DNA library and hybridize to ctDNA (unpublished data). Probes followed by a letter in parentheses are from other laboratories: a, Polans et al. 1986; b, Harper et al. 1989; c, Burke et al. 1988; d, Simon et al. 1985; e, Crouch et al. 1983; f, Chase and Palmer 1989; g, Palmer and Shield 1984

part glacial acetic acid) for 12–24 h. The fixed tissue was then digested for 2 h in a solution containing pectinase and cellulase (1:1) Slides were stained by the Giemsa staining method. For meiotic analysis, young buds of different sizes were harvested at 9:00 to 11:00 a.m. and fixed in Carnoy's solution. Anthers were dissected, stained in 1% acetocarmine staining solution, squashed, and examined under a light microscope. The pollen stainability of hybrids and derived amphidiploids was based on at least 200 mature pollen grains in 1% acetocarmine (Quiros et al. 1988).

Molecular characterization

RFLP analysis was used to verify the hybridity of F_1 plants and their doubled amphidiploids and to compare synthetic lines and natural amphidiploid accessions. Plant DNAs were isolated from lyophilized young leaves or young buds. These DNAs were digested with restriction endonucleases *Eco*RI, *Hin*dIII, or *Bam*HI. Southern blots of these DNA samples were probed with 33 anonymous nuclear DNA clones, 5 cloned nuclear genes of known function, 6 ctDNA clones, and 5 mtDNA clones (Table 2). The methods employed for DNA extraction and RFLP detection are those cited in Song et al. (1988), except that the final washes for the ctDNA and mtDNA probes were in 0.1 × SSC at 60 °C, and for the nuclear DNA probes, in 0.25 × SSC at 60 °C. Random amplified polymorphic DNA (RAPD) markers were generated by polymerase chain reactions (PCR) using six different arbitrary 10-mer oligonucleotides as primers (Williams et al. 1990). PCR were conducted in 20 µl capillary tubes using an air-thermal cycler (Idaho Technology, model 1605). Reactions were in final volumes of 10 µl containing 1 × buffer (50 m*M* TRIS-Cl, pH 8.3, 1 m*M* MgCl, 20 m*M* KCl, and 500 µg/ml BSA), 1 µ*M* primer, 1 unit *Taq* polymerase (Promega), and 25 ng RNase-treated template DNA. The samples were first heated to 94 °C for 4 min, and then the PCR cycling was carried out as follows: 7 s at 92 °C for denaturation, 10 s at 40 °C for annealing, and 60 s at 72 °C for extension. After 40 cycles, the samples were incubated at 72 °C for 6 min. PCR products were analyzed by electrophoresis in 2% agarose gel.

Data analysis

The genomic compositions of synthetic amphidiploids were determined by directly comparing the RFLP patterns of the amphidiploids to those of their parental diploid species. For comparisons between natural and synthetic amphidiploids, 388 nuclear RFLPs were scored as present or absent across all accessions, and data were analyzed by principal coordinate analysis (PCA) using the Jaccard similarity coefficient (NTSYS-pc version 1.40, developed by F. J. Rohlf, State University of New York, Stony Brook).

Results and discussion

Development of synthetic amphidiploids

A total of 16 crosses which included the genome combinations A × B, B × A, A × C, C × A, B × C, and C × B were made among five accessions from the three diploid genomes (Table 3). The selection of parental lines was based on criteria such as fast flowering, no vernalization requirement, and distinct genotypes within *B. rapa* and *B. nigra*. From a preliminary study, we discovered that ovary culture followed by embryo rescue gave the best results for producing interspecific hybrids of *Brassica* (data not shown), and thus this strategy was used for all of the cross combinations. Three different

Table 3. Results from various cross combinations for synthesis of artificial amphidiploids

Cross combination[a]	Number of ovaries cultured	Number of F_1 plants		Number of doubled hybrid plants[b]	Optimum ovary age[c]	Optimum media for ovary culture[d]
		Total	Per ovary			
A1 × B1	79	55	0.70	2	8–10	N1 or B5-1
B1 × A1	155	0	–	–	–	–
A1 × B2	57	10	0.18	1	6–7	W1
B2 × A1	146	1	0.01	0	7–8	N1
A2 × B1*	76	251	3.30	6	10–13	N1
B1 × A2*	33	1	0.03	1	8–10	N1
A2 × B2	94	238	2.50	2	10–11	B5-1 or N1
B2 × A2	178	9	0.05	2	10–11	B5-1
A1 × C	65	29	0.45	3	6–14	N1 or B5-1
C × A1	44	28	0.64	3	10–13	W1
A2 × C*	101	189	1.87	3	6–10	B5-1
C × A2*	58	39	0.67	4	3–12	W1
B1 × C*	73	8	0.10	2	8–9	W1
C × B1*	88	2	0.02	2	13–14	W1
B2 × C	83	10	0.12	3	6–13	W1 or B5-1
C × B2	99	0	–	–	–	–

[a] A1, *B. rapa* cv 'Tobin'; A2, *B. rapa* cv 'Flowering Pak Choi'; B1, *B. nigra*, Ethiopian wild population; B2, *B. nigra* cv 'WBPS'; C, *B. oleracea* CrGC3-1. Combinations with * were selected to advance by self-pollination. The female parent is indicated on the left

[b] Ten F_1 hybrid plants from each of the A × C and C × A combinations, 10–30 plants from each of the A × B combinations, and all hybrid plants from the other cross combinations were treated with colchicine

[c] Optimum ovary age (days after pollination) for ovary culture

[d] N1, Nitch and Nitch's (Sigma); B5-1, B5 (Sigma) with 0.02 mg NAA/l and 0.02 mg IAA/l; W1, White (1963 formula, Sigma) with 300 mg casein hydrolysate/l

culture media and different ovary ages were used for the in vitro culture in order to increase the chance of obtaining hybrids. We found that our choice of ovary age and media for in vitro culture affected our ability to rescue hybrids and that optimum treatments varied among the different genome combinations (Table 3).

Large differences in the efficiency of producing synthetic amphidiploids were observed between reciprocal crosses. Usually, the cross combinations that had cytoplasm donors resembling those of the natural amphidiploids gave much higher yields of hybrids than their reciprocals (Table 3). This was especially true when the reciprocal crosses involved species with highly differentiated cytoplasms, such as A and B or B and C (Palmer et al. 1983; Song et al. 1988). In our study, whenever *B. nigra* (B-type cytoplasm) was used as a parent, it was very difficult to obtain hybrids from crosses that were in the opposite direction of the natural amphidiploids. For instance, the cross combination A2 × B1 produced 251 hybrid plants from 76 cultured ovaries (3.3 plants per ovary), whereas the reciprocal produced only 1 hybrid plant from 33 ovaries (0.03 plants per ovary). A similar situation was observed for the B1 × C and C × B1 cross combinations (Table 3). Reciprocal difference were also observed in the efficiency of chromosome doubling and in the fertility of synthetic amphidiploids. Hybrids having B or C cytoplasms were usually easier to double than hybrids with A cytoplasms (data not shown). These results suggest that the cytoplasm type of the maternal parent played a critical role in the success of producing synthetic amphidiploids.

Hybrids were obtained from 14 of the 16 genome combinations (Table 3). However, for some of the hybrids chromosome doubling was difficult to achieve, especially for those containing genomes from 'Tobin' (A1) or 'WPBS' (B2). Also, we failed to obtain seeds from some of the doubled amphidiploids, even from some plants that had 65–90% stainable pollen. Because of these difficulties, only a subset of the hybrids obtained were selected to advance by self-pollination (Tables 3 and 4).

Morphological and cytological characterization of synthetic amphidiploids

All of the F_1 hybrids and their amphidiploids had the intermediate morphotypes of their parental species for a number of traits, such as leaf and flower characteristics (Fig. 1). However, differences in plant shape, leaf color and shape, and flower size were observed between the reciprocal hybrids of some genome combinations. In all cases, the reciprocal hybrids more closely resembled the maternal parent than the paternal parent (see Fig. 1). All of the F_1 hybrids were highly sterile with 2% or less stainable pollen. After chromosome doubling, the amphidiploids from A × C and C × A easily produced self-pollinated seeds. However, severe embryo abortion was observed after the pollination of amphidiploids from A × B, B × A, B × C, and C × B, although these amphidiploid plants had 45–91% stainable pollen (Table 4) and produced normal siliques. Embryo abortion of these amphidiploids occurred 3–5 days earlier (at the early heart stage) than embryo abortion of their F_1 parental embryos (at the late heart stage to torpedo stage).

The fertility of all of the synthetic amphidiploids was improved slightly in the F_3 generation, and we were able to obtain a few seeds from the genome combinations A × B, B × A, B × C, and C × B. Progenies from the self-pollination of an individual amphidiploid plant had morphotypes (very similar) to each other and to that of their parent, which was

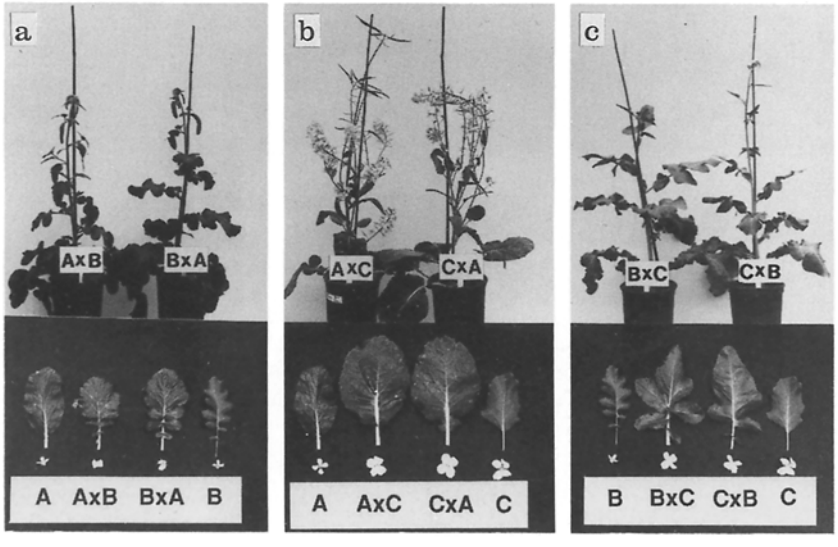

Fig. 1a–c. Morphology of synthetic amphidiploids from reciprocal hybridizations. **a** Amphidiploids from A × B and B × A (*B. juncea*), **b** amphidiploids from A × C and C × A (*B. napus*), and **c** amphidiploids from B × C and C × B (*B. carinata*). The *upper panel* shows the plant shapes, and the *lower panel* shows leaf and flower characteristics. The synthetic amphidiploids have characteristics intermediate to those of their parental diploid species. The reciprocals from A × B/B × A and B × C/C × B have different leaf shapes and resemble more the maternal parents than the paternal parents (see text for details)

Table 4. Cytological characterization of synthetic amphidiploid lines selected for increasing to advanced generations

Line code	Cross combination[a]	Generation tested[b]	Chromosome no. (2n)	Pollen Stainability[c] (%)
002	A1 × C	F_2	38	76–91 (3)
		F_3	38	55–89 (3)
012	C × A1	F_2	38	75–89 (3)
		F_3	38	84–89 (3)
066	A2 × B1	F_2	36	56–91 (3)
		F_3	36	84–89 (2)
071	B1 × A2	F_2	36	82 (1)
		F_3	36	25–83 (2)
221	B1 × C	F_2	34	45–62 (2)
		F_3	34	52–78 (3)
151	C × B1	F_2	34	61–70 (2)
		F_3	34	50–75 (3)

[a] Designations are the same as in Table 3
[b] F_2 plants are self-pollinated progenies of doubled F_1 hybrids
[c] The numbers in parenthesis are the numbers of plants examined

consistent with the expectation that the genetic constitutions of all amphidiploid plants were fixed after chromosome doubling. However, a variation in fertility was observed among individuals obtained from the self-pollination of single plants in both the F_3 and F_4 generations. This instability in fertility appeared to be associated with specific cytoplasm types. In all cases, the synthetic amphidiploids having cytoplasms which were the opposite of those of their natural forms had a much wider range of variation and an average lower fertility than their reciprocals. For example, the synthetic *B. juncea* with the B-type cytoplasm (line 071, F_3 in Table 4) showed a higher level of sterility and greater variation than the synthetic *B. juncea* with the A-type cytoplasm (line 066, F_3), this latter type of cytoplasm being the same as that of natural *B. juncea.* However, all of the lines listed in Table 4 had a very good seed set when crossed as females to the corresponding natural amphidiploids.

Another interesting phenomenon we observed involved the inheritance of self-incompatibility (SI) in some of the synthetic amphidiploids. All of the parental materials we used were self-incompatible except for the C genome parent CrGC3-1, which was self-compatible. The synthetic amphidiploids produced by combining A × C and C × A (lines 002 and 012 in Table 4) were all self-incompatible, and the self-incompatibility was very stable in self-pollinated progenies of these lines. However, the synthetic amphidiploids having genome combinations B × C and C × B (lines 221 and 151 in Table 4) were all self-compatible and produced embryos by open flower self-pollination. These observations indicate that the SI gene from the A genome and SI gene from the B genome were expressed differently when combined with the same C genome, which might be due to the SI alleles from the B and C genomes interacting differently than those of the A and C genomes.

Synthetic amphidiploids were characterized cytologically by mitotic and meiotic analyses. Chromosome counts were conducted using young leaves to confirm the chromosome numbers of F_1 hybrids and their amphidiploids. All of the F_1 hybrids examined (65 plants total) had chromosome numbers that were the sums of their respective diploid parental species, i.e., A × C and C × A hybrids had n = 19, A × B and B × A hybrids had n = 18, and B × C and C × B hybrids had n = 17. F_2 plants from doubled F_1 hybrids usually had the expected numbers of chromosomes (see Table 4), although some plants were aneuploids, having one to three more or fewer chromosomes than the normal amphidiploids. The meiosis of haploid F_1 hybrids varied considerably, depending on the cross combination. In synthetic F_1 *B. napus* lines (A × C and C × A), chromosomes from the A and C genomes paired very well in metaphase I with 0–3 univalents. However, chromosome paring in synthetic *B. juncea* (A × B and B × A) and *B. carinata* (B × C and C × B) F_1 lines was very poor. The numbers of univalents in most pollen mother cells ranged from 7 to 12 for A × B or B × A and 6 to 10 for B × C or C × B. After chromosome doubling, the amphidiploid plants had a much improved chromosome pairing, but still about 10–20% of the pollen mother cells showed abnormal meiosis, including chromosome lagging and chromosome bridges. Some multivalents were also observed in the synthetic amphidiploid lines. These results agree with those reported previously for synthetic amphidiploids (Olsson 1960a,b; Prakash and Hinata 1980).

Molecular characterization of synthetic amphidiploids

Cytoplasmic and nuclear components of the reciprocal hybrids were characterized by RFLP patterns using ctDNA, mtDNA and nuclear DNA clones (Table 2). Inheritance of the chloroplast and mitochondrial genomes of the synthetic amphidiploids was strictly maternal for all synthetic *B. juncea* and *B. carinata* F_1 plants and most of the synthetic *B. napus* F_1 plants. All of the 18 A × B F_1 plants examined showed the A-type ctDNA and mtDNA RFLP patterns, and all 11 B × A hybrid plants examined had the B-type ctDNA and mtDNA RFLP patterns. Nine B × C plants had the B-type ctDNA and mtDNA, and 2 C × B plants had the C-type cytoplasm (see Fig. 2a, b). Ten F_1 plants of the C × A combination had the C-type cytoplasm, and 9 of the 10 A × C F_1 plants had the A-type cytoplasm (see Fig. 2a, b). An exception was observed in 1 of the 10 A × C F_1 hybrid plants. This plant ($003F_1$) had ctDNA and mtDNA RFLP patterns that were the combined

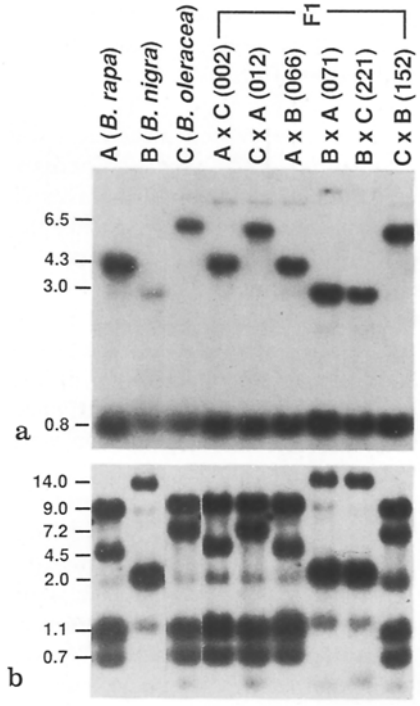

Fig. 2a, b. Autoradiographs showing cytoplasmic RFLP patterns of F_1 hybrids. Designations and line codes are the same as in Table 3. **a** *Eco*RI-digested DNAs probed with the chloroplast DNA clone p8a showing that all of the F_1 hybrids have maternal ctDNA RFLP patterns. **b** *Eco*RI-digested DNAs probed with the mitochondrial DNA clone p4.4 showing that all of the F_1 hybrids have the maternal mtDNA RFLP patterns

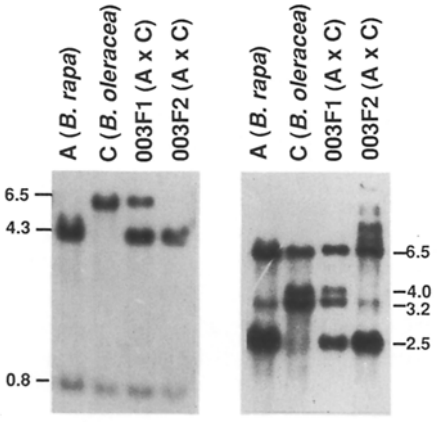

Fig. 3. Autoradiographs showing a hybrid plant, $003F_1$, from the A × C genome combination with the combined RFLP patterns of parental cytoplasms. The *left panel* shows *Eco*RI-digested DNAs probed with ctDNA clone p8a, and the *right panel* shows *Bam*HI-digested DNAs probed with mtDNA clone S10.1 Both ctDNA and mtDNA restriction fragments from the male parent (*C*) are misssing in the F_2 progeny ($003F_2$)

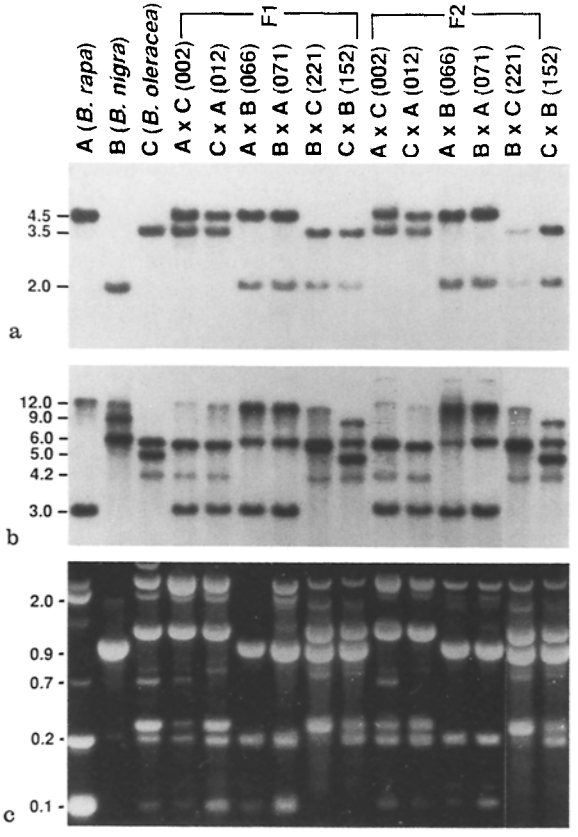

Fig. 4a–c. Genetic characterization of F_1 hybrids and the derived amphidiploids using nuclear RFLP probes and RAPD markers. **a** *Eco*RI-digested DNAs probed with the nuclear DNA clone 161. The diploid parental species A, B, and C are homozygous for the 4.5-kb, 2.0-kb, and 3.5-kb fragments, respectively. The F_1 hybrids and F_2 amphidiploids have the combined patterns of their parents. **b** *Eco*RI-digested DNAs probed with the nuclear DNA clone pC1. The reciprocal hybrids BC and CB have different RFLP patterns, probably because they inherited different allelic fragments from heterozygous parents (see text). **c** PCR products amplified from total genomic DNA using an arbitrary 10-mer oligonucleotide (ATGTCGTATCG). The F_1 hybrids and their derived amphidiploids have the combined patterns of the diploid parents for major bands, although some minor bands are missing

patterns of *B. rapa* and *B. oleracea* (Fig. 3). However, the paternal chloroplast and mitochondrial genomes were absent in the F_2 progeny ($003F_2$). Biparental inheritance of both chloroplasts and mitochondria have been reported in natural *B. napus* (Erickson and Kemble 1990). Our results provide evidence of the biparental inheritance of organelle genomes in an interspecific cross of *B. rapa* × *B. oleracea*. The loss of paternally transmitted organelle genomes in the self-pollinated progeny of $003F_1$ might be due to unequal rates of replication and/or unequal transmission of the original maternal and paternal cytoplasmic genomes.

Nuclear genomes of the synthetic amphidiploids showed combined RFLP patterns of their parental

diploid species for all of the nuclear DNA clones examined (see Fig. 4). Although in most cases the reciprocal hybrids from the same genomic combination showed the same nuclear RFLP patterns (see Fig. 4a), some differences were observed between the reciprocals; these are probably due to the inheritance of different allelic fragments from heterozygous parents. For example, the probe pC1 revealed three fragments of 12 kb, 9.0 kb, and 6.0 kb in the B genome parent, and three fragments of 6.0 kb, 5.0 kb, and 4.2 kb in the C genome parent. The F_1 hybrid from the B × C combination appears to have inherited the 12-kb fragment and one copy of the 6.0-kb fragment from B and the 4.2-kb fragment and one copy of the 6.0-kb fragment from C, whereas the reciprocal hybrid from C × B probably inherited the 9.0- and 6.0-kb fragments from B and the 5.0-kb and 4.2-kb fragments from C (Fig. 4b). A combined genome composition of synthetic amphidiploids also was observed using six primers to detect RAPD markers (see Fig. 4c). The RAPD markers detected extensive genetic diversity among parental lines and may be useful in a large-scale search for rapid genome changes in synthetic amphidiploids. The genotyping of F_1 and F_2 plants indicated that the nuclear genomes of synthetic amphidiploids were fixed after chromosome doubling of the F_1 hybrids (Fig. 4). Therefore, if any changes in the genome composition of synthetic amphidiploids are detected in subsequent generations, they should be due to genome rearrangements rather than the segregation of heterozygous markers.

Comparison between natural and synthetic amphidiploids

The genomic compositions of synthetic lines and natural diploid and amphidiploid accessions were compared using ctDNA, mtDNA, and nuclear DNA RFLPs. All of the *B. carinata* accessions examined had *B. nigra* (B type) cytoplasm and all of the *B. juncea* accessions had *B. rapa* (A type) cytoplasm (Table 1). These results are in agreement with previous characterization of chloroplast genomes of these species (Erickson et al. 1983; Palmer et al. 1983). Four types of cytoplasms were found for the natural accessions of *B. napus*, suggesting that *B. napus* originated from multiple hybridization events (Table 1; Song and Osborn 1992).

Principal coordinate analysis of nuclear RFLP data revealed that most of the synthetic amphidiploids were more closely related to their progenitor diploid species than the natural amphidiploids (Fig. 5). Reciprocal hybrids of a given genome combination were clustered together and usually were equidistant to the two diploid parental lines. Accessions within each of the natural amphidiploids were clustered together, however the clusters were positioned far from their respective synthetic amphidiploid and, with the exception of *B. carinata*, they were much farther from their hypothesized diploid progenitor species than the synthetics. Large differences between natural amphidiploids and our synthetic amphidiploids suggest that the natural amphidiploid species have changed greatly since their synthesis from the diploid species and/or that the diploid parents of natural amphidiploids were very different from the diploid parents used in the synthesis of our artificial amphidiploids.

Conclusions

This paper represents the first report of a complete set of synthetic *Brassica* amphidiploids derived by reciprocal hybridizations. This set of amphidiploids includes all possible nuclear-cytoplasmic combinations,

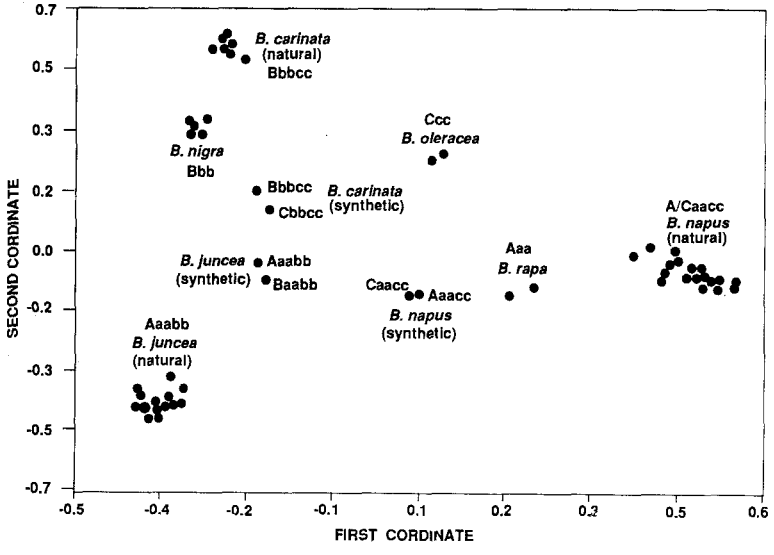

Fig. 5. A two-dimensional illustration based on principle coordinate analysis of nuclear RFLP data showing genetic relationships between synthetic and natural amphidiploids and their diploid parental species. All of the synthetic amphidiploids are more closely related to the diploid species than the natural amphidiploids

and it also includes combinations of nuclear genomes that are closely related (*B. napus*) and those that are more distantly related (*B. juncea* and *B. carinata*). Morphological, cytological, and molecular analyses have confirmed the hybrid nature of the nuclear genomes and the maternal origin of the cytoplasmic genomes. Although these amphidiploids should be completely homozygous following chromosome doubling, preliminary results on pollen stainability and meiotic behavior suggest that progeny from all of the synthetic amphidiploids are genetically variable. Also, nuclear RFLP data showed that most of the natural amphidiploids are much more divergent from their diploid progenitors than the synthetic ones. These observations suggest that the natural amphidiploids may have changed substantially after their synthesis, and that genome changes leading to the genetic stabilization of newly formed amphidiploids may occur in early generations after synthesis. Our synthetic amphidiploids provide an excellent system for investigating the potential for rapid genome evolution in amphidiploids. Using various molecular methods, we can determine the degree and timing of changes in genome structure and gene expression and the possible role of nuclear-cytoplasmic interactions in this process.

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