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Interspecific hybridization in *Brassica*: Application of flow cytometry for analysis of ploidy and genome composition in hybrid plants

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Abstract

Interspecific hybrids from the crosses between *Brassica campestris, B. carinata, B. juncea* and *B. napus* were obtained through *in vitro* ovary and ovule culture. F_1 hybrids were studied morphologically and flow cytometry was used to estimate 2C nuclear DNA content both in parental *Brassica* species and their hybrids. It was found that in comparison with the A genome, the B and the C genomes of *Brassica* contained 26.9 % and 43.9 % more DNA, respectively. This finding may be used to distinguish interspecific hybrids containing various genome combinations. It was concluded that flow cytometric analysis of nuclear DNA content might be useful tool in *Brassica* breeding.

Introduction

Rapeseed (*Brassica napus* L. and *Brassica campestris* L.) and mustard (*Brassica juncea* Coss., *Brassica nigra* L., *Brassica carinata* A. Braun, and *Brassica hirta* Moench), members of the family *Cruciferae* are herbaceous annuals closely related botanically each other and are important oilseed crops. On the basis of cytological analysis of chromosome pairing, Morinaga (1934) put forward the hypothesis that three species with higher chromosome numbers, *B. napus* (2n = 38), *B. juncea* (2n = 36) and *B. carinata* (2n = 34) originated as amphidiploid hybrids from the combinations of monogenomic or diploid species, *B. nigra* (2n = 16, BB), *B. campestris* (2n = 20, AA) and *B. oleracea* (2n = 18, CC). This hypothesis was verified by U (1935) successfully synthesizing *B. napus* from crosses between diploid *B. campestris* and *B. oleracea.*

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The knowledge of relationships between different *Brassica* species has created possibilities of utilizing interspecific crosses in the breeding programmes aimed at the production of new resynthetic rape material and at the transfer of genes between diploid and polyploid *Brassica* species. However, in some cases, it is rather difficult to obtain interspecific hybrids due to genetic imbalance of parents. Distant crosses may fail because of endosperm degeneration which blocks the normal development of young embryo and may lead to its death. In order to enhance the efficiency of wide hybridization various *in vitro* techniques, such as embryo culture (Ross 1980), ovary culture (Inomata 1979), ovule culture (Matsuzawa 1978) and protoplast culture (Sundberg *et al.* 1987) have been employed. *In vitro* culture techniques not only accelerate the process but may be also used to induce genetic variability (Larkin and Scowcroft 1981) which may be of great importance to a breeder.

Flow cytometry may be used for rapid and convenient analysis of nuclear DNA content (Brown *et al.* 1991, Doležel 1991). The method entails isolation of intact nuclei and their staining by a DNA specific fluorochrome. The fluorescence of stained nuclei is then analysed using a flow cytometer. Flow cytometric analysis of nuclear DNA content in plants has proved to be useful for instance in studies of interspecific and intraspecific DNA content variation in plants (Michaelson *et al.* 1991, Hammat *et al.* 1991) and in determination of ploidy in single plants and plant populations (De Laat *et al.* 1987).

Here we report on production of interspecific hybrids between *B. campestris, B. juncea, B. napus* and *B. carinata* using *in vitro* culture. Hybrid plants were studied morphologically. Flow cytometry was used to estimate nuclear DNA content in parental plants and their hybrids. We have found that the basic three *Brassica* genomes (A, B, C) differ in size. This allowed to distinguish interspecific hybrids containing various genome combinations.

Materials and methods

Plant material:

The list of parental species and lines used in this study is as follows:

1. *Brassica napus* (2n = 38, AACC)
   Line 250A - represents the fifteenth generation of cross *B. oleracea* cv. Inka. × *B. campestris* cv. Yellow Sarson. It has large and light yellowish brown seeds.
   Line 250B - same as 250A, however, the seeds are small and brownish yellow.
   (Both lines were selected by Dr. M. Bechyně, University of Agriculture, Prague, Czech Republic)
2. *Brassica campestris* (2n = 20, AA):
   Line 9a - yellow seeds
3. *Brassica juncea* (2n = 36, AABB)
   cv. Kikarashina - yellow seeds
   (obtained from Dr. M. Takada, Gifu University, Japan)
4. *Brassica carinata* (2n = 34, BBCC):
   Line 21 - yellow seeds
   (selected by Dr. M. Bechyně)

Interspecific hybridization: Parental plants were grown in a greenhouse. Healthy unopened buds one day prior to anthesis were selected, hand emasculated and pollinated with the pollen of desired lines. The crossed buds were bagged for isolation. All the crosses were made in the morning between 08.00 and 10.00 local time.

***In vitro* culture:** Seven days after pollination, the crossed pods were excised and cultured on MS medium (Murashige and Skoog 1962) under aseptic conditions. Seven days after culturing the pods on the MS medium young ovules were excised and again cultured on the MS medium supplemented with 0.2 mg l^{-1} kinetin and 500 mg l^{-1} casein hydrolysate (Bajaj *et al.* 1986). Young seedlings thus obtained, were transfered to a sterile perlite and grown for ten days. Subsequently, the plantlets were transferred to a soil and grown to maturity in greenhouse.

Isolation and staining of nuclei: Cell nuclei were isolated from young leaves of plants at early bud stage. Approximately 50 mg of leaf tissue was chopped with a sharp scalpel in a glass Petri dish containing 1 ml LB01 lysis buffer (Doležel *et al.* 1989) of the following composition: 15 mM Tris, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 0.1 % Triton X-100, pH 7.5, supplemented with 50 μg propidium iodide and 50 μg RNase. To compare nuclear DNA content of two different genotypes, approximately 25 mg of leaf tissue of each genotype was chopped simultaneously. The suspensions of released nuclei were passed through a 50 μm nylon mesh and stained in the dark for 30 min. Then the samples were filtered through a 15 μm nylon mesh and analysed. The whole procedure was performed on ice.

Flow cytometry: Propidium iodide-stained nuclei were analysed with a Leitz MPV-Compact Flow cytometer. A filterblock N2 was used both for the excitation and for the detection of propidium iodide fluorescence. Histograms of fluorescence intensity were registered over 512 channels and evaluated using a *Hewlett-Packard HP-86B* microcomputer with a *FLOWSTAR* software (Doležel 1989). At least ten thousand nuclei were analysed in each sample. Each sample was analysed five times.

Data analysis: The 2C nuclear DNA content was expressed in arbitrary units (A.U.). *Brassica campestris* was defined as having 100 A.U. The ratio of means of the G_0/G_1 peaks corresponding to the reference standard and sample nuclei (fluorescence ratio) was calculated. The ratio was corrected for zero offset error using the DNA ratio between single and double chicken red blood cell nuclei clumps (Givan *et al.* 1988). Relative 2C nuclear DNA content of each plant was calculated according to a formula:

$$\text{2C nuclear DNA content (A.U.)} = \frac{100}{\text{fluorescence ratio}}$$

Results

While a cross *B. napus* x *B. campestris* produced 0.52 seeds per pollination, a reverse cross produced 3.62 seeds per pollination (Table 1). Similarly, more hybrid seeds were obtained when *B. napus* was used as a pollinator in cross between *B. juncea* and *B. napus*.

Table 1. Results of crosses made within the genus *Brassica*.

Cross	Buds crossed	Obtained pods	F_1 seeds	Seeds per pollination	Hybrid seed colour
B. napus x *B. campestris*	109	109	57	0.52	yellowish brown
B. campestris x *B. napus*	50	50	181	3.62	yellowish brown
B. napus x *B. juncea*	95	44	14	0.32	brown
B. juncea x *B. napus*	87	71	56	0.79	yellow
B. carinata x *B. napus*	44	44	2	0.045	yellow

Morphology of F_1 hybrid plants.

1) *B. napus* x *B. campestris*. Leaf shape, size and colour in F_1 hybrids was similar to that of *B. napus*. Trichomes on leaf surface were absent in all cases. Petals of F_1 hybrids were intermediate in size and pale yellow in colour. Stamens and anthers were well developed. Anthocyanin spot which is present in *B. napus* and absent in *B. campestris* was also absent in F_1 hybrids.

2) *B. campestris* x *B. napus*. Leaf shape and size was similar to that of *B. napus*, leaf margin being undulating. Leaf thickness of F_1 hybrids was less in comparison with *B. napus*. Anthocyanin was present on the stem leading towards the leaf petiole and leaf margin. Petal shape, size and colour resembled that of *B. napus*.

3) *B. napus* x *B. juncea*. Leaf morphology was similar to that of *B. napus*. Trichomes which are present on both sides of leaves in *B. juncea* and absent in *B. napus* were also absent in F_1 hybrids. Floral morphology of F_1 hybrid was intermediate between two parents. Anthers were rudimentary and hidden in petals almost without pollen grains. Anthocyanin line demarking the carpels on the pods which is present in *B. napus* and absent in *B. juncea* was observed in F_1 hybrids.

4) *B. juncea* x *B. napus*. Architecture of F_1 plants was similar to that of *B. juncea*. Leaf and stem morphology of F_1 hybrid plants was dominated by *B. juncea* characters. Stamens and anthers were well developed, however, large proportion of pollen grains were not fertile.

5) *B. carinata* x *B. napus*. Strong dominance of *B. carinata* characters in F_1 hybrids was observed. In some cases multi-stemmed plants were found. Petal shape, size and colour was intermediate between the parents. The flowers of F_1 hybrids were male sterile, stamens were rudimentary, sticking to the base of pistil, almost without pollen grains.

Estimation of relative nuclear DNA content: Flow cytometric analysis of propidium iodide-stained nuclei isolated from *B. campestris* showed that more than 85 % of nuclei were in G_0/G_1 phase of the cell cycle (Fig. 1). Similar distributions of relative nuclear DNA content were also found in other *Brassica* species and their hybrids. These distributions were shifted more to the right which indicated higher DNA content. The coefficients of variation of G_0/G_1 peaks ranged between 3 % and 5 % throughout this study.

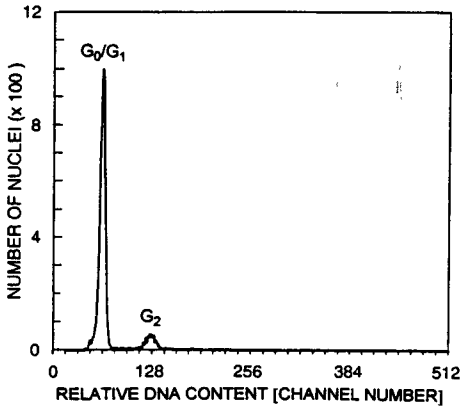

Fig. 1. Histogram of relative DNA content of nuclei isolated from young leaf of *B. campestris* cv. Yellow Sarson. Most of nuclei are in G_0/G_1 phase of the cell cycle and form a large peak at channel 64.

The results of the analysis of relative 2C nuclear DNA content in *Brassica* species used for interspecific hybridization are summarized in Table 2. With the DNA content of *B. campestris* defined as 100 A.U., and using the data obtained in *B. napus* and *B. juncea*, relative sizes of the B and C genomes were calculated to be 63.46 A.U. and 71.95 A.U., respectively. Thus in comparison with the A genome, the B and the C genomes of *Brassica* contain 26.9 % and 43.9 % more DNA,

Table 2. Relative 2C nuclear DNA content of *Brassica* species.

Species	2n	Genome	DNA content (A.U.)
B. campestris	20	AA	100
B. juncea	36	AABB	226.9
B. napus	38	AACC	243.9
B. carinata	34	BBCC	258.1

respectively. The relative DNA content estimated in *B. carinata* was similar to that calculated as a sum of two diploid sets (BB + CC). The difference was less than 5 %. An example of nuclear DNA content analysis in *B. napus* is shown in Fig.2.

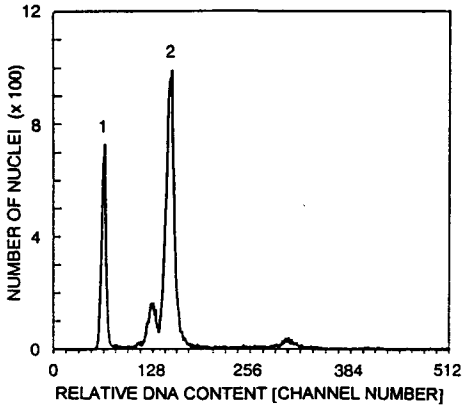

Fig. 2. Distribution of relative DNA content of nuclei isolated *B. campestris* cv. Yellow Sarson and *B. napus* line 250A. The nuclei were isolated, stained and analysed simultaneously. The first peak on the left (1) represents G_0/G_1 nuclei of *B. campestris* while the largest peak (2) represents G_0/G_1 nuclei of *B. napus*.

Table 3. Relative 2C nuclear DNA content of *Brassica* interspecific hybrids and their probable genome composition*.

Cross	Plant	DNA content (A.U.)	Genome composition
B. napus x *B. campestris*	NCM01	215.3	AAAc
	NCM02	178.4	AAC
	NCM03	174.0	AAC
	NCM04	170.3	AAC
B. campestris x *B. napus*	CMN01	181.5	AAC
B. napus x *B. juncea*	NJ01	236.0	AABC
	NJ02	358.1	AAABBC
B. juncea x *B. napus*	JN01	167.5	AAB
	JN02	239.2	AABC
	JN03	231.3	AABC
	JN04	236.6	AABC
B. carinata x *B. napus*	CRN01	252.7	ABCC

*Estimated on the basis of nuclear DNA content.

A summary of relative 2C nuclear DNA content estimated in interspecific *Brassica* hybrids obtained in this study is given in Table 3. Because the A, B and the C genomes of *Brassica* differ in size, 2C nuclear DNA content of F_1 hybrids was

used not only to estimate their ploidy but also to estimate their genome composition. An example of the flow cytometric analysis of nuclear DNA content in an interspecific hybrid is shown in Fig. 3.

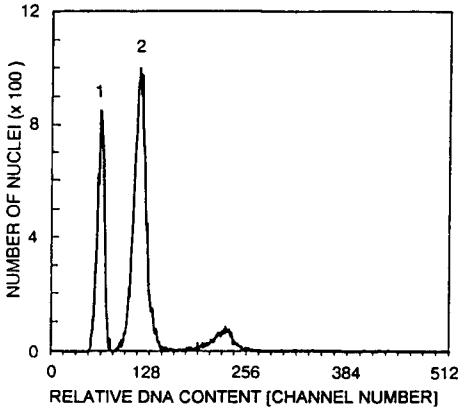

Fig. 3. Relative DNA content distribution of nuclei isolated from *B. campestris* cv. Yellow Sarson and F_1 hybrid *B. napus* x *B. campestris* (NCM02). The nuclei were isolated, stained and analysed simultaneously. The first peak on the left (1) represents G_0/G_1 nuclei of *B. campestris* while the second peak (2) represents G_0/G_1 nuclei of the F1 hybrid.

Discussion

A number of reports described the use of *in vitro* embryo, ovary and ovule culture to obtain *Brassica* hybrids: *B. campestris* x *B. oleracea* (Inomata 1979, Takeshita *et al.* 1980), *B. fruticulosa* x *B. campestris* (Nand Kumar *et al.* 1988), *B. napus* x *B. juncea* (Bajaj *et al.* 1986) and *B. juncea* x *B. hirta* (Mohapatra and Bajaj 1985). *B. napus* crosses easily with *B. campestris* and *B. juncea* by conventional methods, however, *in vitro* culture is also employed to increase genetic variability which could be usefull in *Brassica* programmes. The occurrence of multi-stemmed plants could be explained by the influence of culture medium on plants grown *in vitro*. We have obtained partly fertile hybrids from the cross *B. napus* x *B. juncea*, while Bajaj *et al.* (1986) obtained fertile hybrids. Partly fertile hybrids were also obtained in the cross *B. napus* x *B. carinata* (Aslam and Bechyně 1982). Their decreased fertility may be due to the chromosomal disharmony between two genomes.

Our results suggest that despite of having less chromosomes, *B. juncea* and *B.campestris* proved to be better maternal parents producing more seeds in comparison with *B. napus*. This is in accordance with the Olsson's contention (Olsson 1960) that genotype and physiological status have greater influence on crossing than the number of chromosomes.

We have shown that flow cytometry is a suitable technique for rapid analysis of nuclear DNA content in *Brassica* species and their hybrids. Because nuclear DNA

content of a given genome is proportional to the number of haploid chromosome sets it contains, flow cytometry may be used to estimate ploidy level. This was clearly demonstrated by De Laat *et al.* (1987) who analysed ploidy of single plant and plant populations of sugarbeet.

In alloploids, which contain two or more basic genomes of different sizes, the nuclear DNA content depends not only on the ploidy but also on the actual genome composition. Our results suggest that this is the situation in *Brassica*. We have found that the A, the B and the C genomes of *Brassica* differ considerably in size. Similar differences were reported by Verma and Rees (1974) who analysed DNA content in *Brassica* species by Feulgen microdensitometry. Thus in alloploids, a care should be taken when interpreting the results of flow cytometric DNA content estimation in terms of chromosome number.

Interestingly, Fahelson *et al.* (1988) who compared nuclear DNA content and chromosome number in somatic hybrids within *Brassicaceae*, concluded that flow cytometry could be used for rapid estimation of chromosome number. The disagreement of this conclusion with the results of this study may be explained by a lower accuracy of chromosome number estimation reported by the authors (± 10 %). Such variation could hide the differences caused by the effect of various genome sizes of parental species. In fact, the authors noted the presence of hybrids having different chromosome number and the same DNA content.

To conclude, we have shown that flow cytometry is useful tool for estimation of ploidy and genome composition in *Brassica*. Because the technique is convenient and rapid it may become a useful tool in *Brassica* breeding.

References

Aslam Y, Bechyně, M.: [Morphology and cytology of F_1 hybrids between *Brassica napus* and *Brassica carinata* A. Braun.] - Sbor. VŠZ Praze, Fak. Agron. **38**: 243-253, 1983. [In Czech.]

Bajaj, Y.P.S., Mahajan, S.K., Labana, K.S.: Interspecific hybridization of *Brassica napus* through ovaries, ovules and embryo culture. - Euphytica **35**: 103-109, 1986.

Brown, S.C., Bergounioux, C., Tallet, S., Marie, D.: Flow cytometry of nuclei for ploidy and cell cycle analysis. - In: Negrutiu, I., Gharti-Ghhetri, G. (ed.): A Laboratory Guide for Cellular and Molecular Plant Biology. Pp. 327-345. Birkhäuser, Zürich 1991.

De Laat, A.M.M., Göhde, W., Vogelzang, M.J.D.C.: Determination of ploidy of single plants and plant populations by flow cytometry. - Plant Breeding **99**: 303-307, 1987.

Doležel, J.: FLOWSTAR: A microcomputer program for flow cytometric data manipulation and analysis. - Biológia (Bratislava) **44** : 287-291, 1989.

Doležel, J.: Flow cytometric analysis of nuclear DNA content in higher plants. - Phytochem. Anal. **2**: 143-154, 1991.

Doležel, J., Binarová, P., Lucretti, S.: Analysis of nuclear DNA content in plant cells by flow cytometry. - Biol. Plant. **31**: 113-120, 1989.

Fahleson, J., Dixelius, J., Sundberg, E. Glimelius, K.: Correlation between flow cytometric determination of nuclear DNA content and chromosome number in somatic hybrids within *Brasicaceae*. - Plant Cell Rep. **7**: 74-77, 1988.

Givan, A.L., Shenton, B.K., Carr, T.W.: A correction required for calculation of DNA ratios in flow cytometric analysis of ploidy. - Cytometry **9**: 271-274, 1988.

Hakanson, A.: Seed development of *Brassica oleracea* and *Brassica rapa* after certain reciprocal

pollinations. - Hereditas **42**: 373-396, 1956.

Hammatt, N., Blackhall, N.W., Davey, M.R.: Variation in the DNA content of *Glycine* species. - J. exp. Bot. **42**: 659-665, 1991.

Inomata, N.: Production of interspecific hybrids in *Brassica campestris* × *Brassica oleracea* by culture *in-vitro* of excised ovaries. II. Development of excised ovaries on various culture media. - Jap. J. Breed. **29** :115-120, 1979.

Larkin, P.J., Scowcroft, W.R.: Somaclonal variation: a novel source of variability from cell cultures for plant improvement. - Theor. appl. Genet. **60**: 197-214, 1981.

Matsuzawa, Y.: Studies on the interspecific hybridization in the genus *Brassica*. I. Effect of temperature on the development of hybrid embryos and improvement of crossability by ovary culture in interspecific cross, *B.campestris* x *B. oleracea*. - Jap. J. Breed. **28**: 186-196, 1978.

Michaelson, M.J., Price, H.J., Johnston, J.S., Ellison, J.R.: Variation of nuclear DNA content in *Helianthus annuus* (*Asteraceae*). - Amer. J. Bot. **78**: 1238-1243, 1991.

Mohapatra, D. Bajaj, Y.P.S.: Interspecific hybridization in *Brassica juncea* × *Brassica hirta* using embryo rescue. - Euphytica **36**: 321-326, 1985.

Morinaga, T.: Interspecific hybridization in brassicas. VI. Cytology of F_1 hybrids of *B. juncea* and *B. nigra*. - Cytology **6**: 63-67, 1934.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nand Kumar, P.B.A., Shivanna, K.R. Prakash, S.: Wide hybridization in Brassicas. Crossability barriers and studies on the F_1 hybrid and synthetic amphidiploid of *B. fruticulosa* × *B. campestris*. - Sex. Plant Rep. **1**: 234-239, 1988.

Olsson, G.: Species cross within the genus *Brassica*. II. Artificial *Brassica napus* L. - Hereditas **46**: 351-396, 1960.

Ross, C.L.: Embryo culture in the production of disease resistant brassicas. - In: Ingram, D.S., Helgeson, J.P. (ed.): Tissue Culture Methods for Plant Pathologists. Pp. 225-258. Pentlandfield Scottish Plant Breeding Station, Pentland 1980.

Sundberg, E., Landgren, M. Glimelius, K.: Fertility and chromosome stability in *Brassica napus* resynthesized by protoplast fusion. - Theor. appl. Genet. **75**: 96-104, 1987.

Takeshita, M., Kato, M. Tokumashu, S.: Application of ovule culture to the production of intergeneric or interspecific hybrids in *Brassica* and *Raphanus*. - Jap. J. Genet. **55**: 373-387, 1980.

U, N.: Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. - Jap. J. Bot. **9**: 389-452, 1935.

Verma, S.C., Rees, H.: Nuclear DNA and the evolution of allotetraploid *Brassicae*. - Heredity **33**: 61-68, 1974.

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