

## Construction of intergeneric somatic hybrids between *Brassica oleracea* and *A Armoracia rusticana*

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### Abstract

Somatic hybridization of *Brassica oleracea* (cauliflower, cabbage, kohlrabi) and *A Armoracia rusticana* (horseradish) protoplasts was performed to test the possibility of the formation of intergeneric hybrids. Out of three *B. oleracea* crops tested only cauliflower (*B. oleracea* var. *botrytis*) yielded viable hybrids when fused with horseradish protoplasts. Regenerated plants were asymmetric hybrids as revealed by morphological, karyological, and DNA analysis. In these plants, extensive elimination of *A. rusticana* genome occurred during subsequent cultivation. Using a chloroplast DNA probe no *A. rusticana*-specific hybridization signals were detected thus indicating a rapid elimination of horseradish cytoplasmic (chloroplast) components.

*Additional key words:* asymmetric hybrid, cauliflower, horseradish, protoplast fusion.

### Introduction

Gene transfer between different genera is difficult to achieve by means of classical breeding although in *Brassicaceae* an amphidiploid sexual hybrid of *Raphanus sativus* and *Brassica oleracea* is known for more than 70 years. More recently, "*Raphanobrassica*" hybrid served as a source of cytoplasmic male sterility (Bannerot *et al.* 1974). The resistance to *Alternaria brassicae* has been transferred to *B. napus* using a sexual hybridization with *Sinapis alba* (Ripley and Arnison 1990). Parasexual intergeneric hybrids in *Brassicaceae* (Gleba and Hoffmann 1978, 1980) were shown to be viable and many hybrids have been constructed *via* protoplast fusion to transfer useful traits to hybrid plants, *e.g.*, the resistance to plant pathogens.

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As relatively high levels of resistance to clubroot (*Plasmodiophora brassicae*) infection are known in *Raphanus sativus* (Ashizawa *et al.* 1980, Rod 1993a), this species was used as the source of clubroot resistance in *R. sativus* × *B. oleracea* somatic hybrids (Yamanaka *et al.* 1992). Clubroot has never been observed in horseradish (*Armoracia rusticana*) and this crop seems highly immune to primary infection. As the racial specificity of *Plasmodiophora* isolates is rather underestimated, the extended genetic diversity of resistance resources is highly desirable. In this paper the preliminary results of our experiments on the construction and analysis of intergeneric somatic hybrids between *B. oleracea* and *A. rusticana* are described.

## Material and methods

**Plants:** Seeds of cauliflower line cv. OL-H-66 (*Brassica oleracea* var. *botrytis*,  $2n = 18$ ), cabbage (*B. oleracea* var. *capitata*,  $2n = 18$ ) and kohlrabi (*B. oleracea* var. *gongylodes*,  $2n = 18$ ) were obtained from the vegetable germplasm collection of the Research Institute of Crop Production, Prague. Cauliflower cultivar Fastman was kindly provided by Bejo Zaden (Warmenhuizen, The Netherlands). Horseradish (*Armoracia rusticana*,  $2n = 32$ ) was a clonal offspring of a plant raised from true seed harvested from the landrace Malínský.

**Protoplast preparation:** Horseradish mesophyll protoplasts were isolated from fully developed leaves of plants cultivated *in vitro* on hormone-free Murashige and Skoog (MS) medium. Cultures were kept at 24 °C, 16 h photoperiod (irradiance of 95  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). To isolate *Brassica* hypocotyl protoplasts, seeds of *B. oleracea* were sterilized and cultured for 7 d in the dark at 25 °C on a half concentrated MS medium. Leaves and hypocotyls were chopped and overlaid by filter-sterilized enzyme solution containing 1 % of cellulase *Onozuka OR 10* (Serva, Heidelberg) and 0.25 % macerozyme *R 10* (Serva, Heidelberg), and incubated for 18 h in the dark at 25 °C. Crude protoplast suspension was stepwise purified by filtration (polyamide sieve, 72  $\mu\text{m}$ ) and repeated sedimentation (100 g for 5 min) followed by washing in W5 medium (Potrykus and Shillito 1986). Purification was completed by flotation on 20 % saccharose and the suspension was diluted to the density of  $10^6$  protoplasts per  $\text{cm}^3$ .

**Induction of protoplast fusion:** Leaf mesophyll protoplasts of *A. rusticana* were fused with etiolated hypocotyl protoplasts of three *B. oleracea* crops: *B. oleracea* var. *botrytis* (cauliflower), *B. Oleracea* var. *capitata* (cabbage), and *B. oleracea*, var. *gongylodes* (kohlrabi). The protoplasts of both species were mixed in the 1:1 ratio to the final concentration of  $10^5$  protoplasts per  $\text{cm}^3$ . The fusion was performed using polyethylene glycol (PEG 6000, Serva, Heidelberg) according to Christey *et al.* (1991).

**Plant regeneration:** For the cultivation of protoplasts, the culture media B, C, D, E, F (Pelletier *et al.* 1983) with minor modifications were used sequentially. Protoplast mixture in Petri dishes was kept at 25 °C for 72 h in the dark. After this period the

cultures were maintained in 16/8 h light/dark cycles. After 10 d of initial cultivation the liquid C medium (1 cm<sup>3</sup>) was added to each Petri dish and the cultivation continued for the following 20 d. This incubation period was followed by 3 weeks' cultivation in liquid D medium and 3 weeks in liquid E medium. During this incubation, microcalli appearing in the culture were transferred onto the solid medium F. Regenerated shoots from each microcallus were transferred separately onto MS medium supplemented with 0.1 mg dm<sup>-3</sup> 3-indole butyric acid (IBA) and 0.1 mg dm<sup>-3</sup> 6-benzylamino purine (BAP). All the regenerants derived from the same original microcallus were designed as a line. Rooted plantlets were transferred to the greenhouse.

**Karyological analysis:** For a preliminary check of the chromosomal composition of hybrid plants, the tips of the youngest leaves treated by para-dichlorobenzene for 4 h at room temperature were fixed in ethanol: acetic acid (3:1 v/v). Squashed preparations were stained in Schiff reagent. To verify the chromosomal constitution of *in vitro* cultured hybrid plants root tip metaphases were arrested with 2 mM hydroxy-quinoline for 30 min at room temperature, followed by the incubation at 0 °C for 6 h. Root tips were then fixed in ethanol:acetic acid (3:1, v/v) mixture. Fixed root tips were briefly washed in water and incubated in the enzymatic mixture (Veuskens *et al.* 1995) for 1 h at 37 °C. Enzymatic mixture was changed for 45 % acetic acid and the meristems were squashed onto *Vectabond* (Vector, Burlingame, USA) treated slides. Preparations were stained with 5 % Giemsa solution (Merck, Darmstadt, Germany).

**Southern hybridizations:** Total DNAs of parental as well as of hybrid plants were prepared according to Dellaporta *et al.* (1983). DNAs were digested by *EcoRI* restriction endonuclease, fragments separated on 1 % agarose gel and blotted onto *Hybond N* (Amersham, Little Chalford, England) membrane according to Sambrook *et al.* (1989). As a nuclear DNA probe an internal, 2478 bp long, *EcoRI* fragment of the 25S-rDNA gene isolated from tomato (Kiss *et al.* 1989) was used. Chloroplast DNA, clone pTB18, isolated from tobacco cpDNA (Sugiura *et al.* 1986) was used as a probe for the assessment of the proportion of parental cytoplasm in hybrids and, in control experiments, to check the completeness of digestion of total genomic DNA according to Fajkus and Reich (1991). The DNA probes were labelled with [<sup>32</sup>P] dATP using Rediprime DNA labelling kit (Amersham) and hybridized to the membranes according to Sambrook *et al.* (1989).

## Results

**Protoplast fusion:** Small horseradish protoplasts containing green chloroplasts were unable to regenerate under the given experimental conditions when unfused (Table 1), while the regeneration of etiolated, colourless *Brassica* protoplasts was relatively frequent (at least in cauliflower) thus enabling the discrimination of fusion products from non-fused horseradish protoplasts or horseradish auto-fusions. Fusion

products were inoculated into the liquid medium and visible microcalli were detected after 3 weeks of cultivation. The fusion experiments with kohlrabi protoplasts were unsuccessful and the development of cabbage microcalli was blocked in early stage (Table 1). Calli from fusion experiments with cauliflower (mean size up to 1 mm) were transferred onto the solid medium after 6 weeks. Two types of calli were observed: regenerating calli with centres of embryogenic tissues and white non-embryonic calli (Fig. 1a). The former ones developed small purple meristematic areas which elongated after several weeks and differentiated into regenerated shoots (Fig. 1b). No purple areas were detected in calli derived from previous experiments with control cauliflower protoplasts (data not shown).

Table 1. Results of fusion experiments with *Brassica oleracea* and *Armoracia rusticana* protoplasts.

<i>Brassica oleracea</i> var.	Number of fusion experiments	successful experiments*	Occurrence of				Phenotype
			microcalli	developing microcalli	regenerating calli	complete plants	
<i>botrytis</i>	26	10	+	+	+	+	Purple meristematic areas
<i>capitata</i>	20	1	+	+	-	-	Green calli, necrosis
<i>gongylodes</i>	11	0	-	-	-	-	

\*at least one microcalus per plate occurred

In fusion experiments with horseradish and cauliflower protoplasts out of 519 microcalli obtained, 212 calli developed, but only 83 calli regenerated shoots (Table 2). Finally, 89 regenerated plants were isolated and further analyzed.

Table 2. Comparison of fusion in experiments with two cauliflower genotypes

<i>Brassica oleracea</i> var. <i>botrytis</i>	Number of fusion experiments	successful experiments*	microcalli	developing microcalli	regenerating calli	presence of regenerants**
line OLH 66	11	5	385	154	67	++
cv. Fastman	15	5	134	58	16	+

\*\*regeneration of plants not finished at present

**Morphological variation of regenerated plants:** All regenerated plants resembled the cauliflower type and no horseradish-like regenerants were found. During *in vitro* culture the regenerating shoots were purple and vitreous in appearance. After 2 - 5 passages the plants displayed almost normal cauliflower morphology except that the margins of leaves or petioles were purple-coloured indicating prominent antocyanescent shade of hybrid plants (Fig. 1c). Under the greenhouse conditions, the

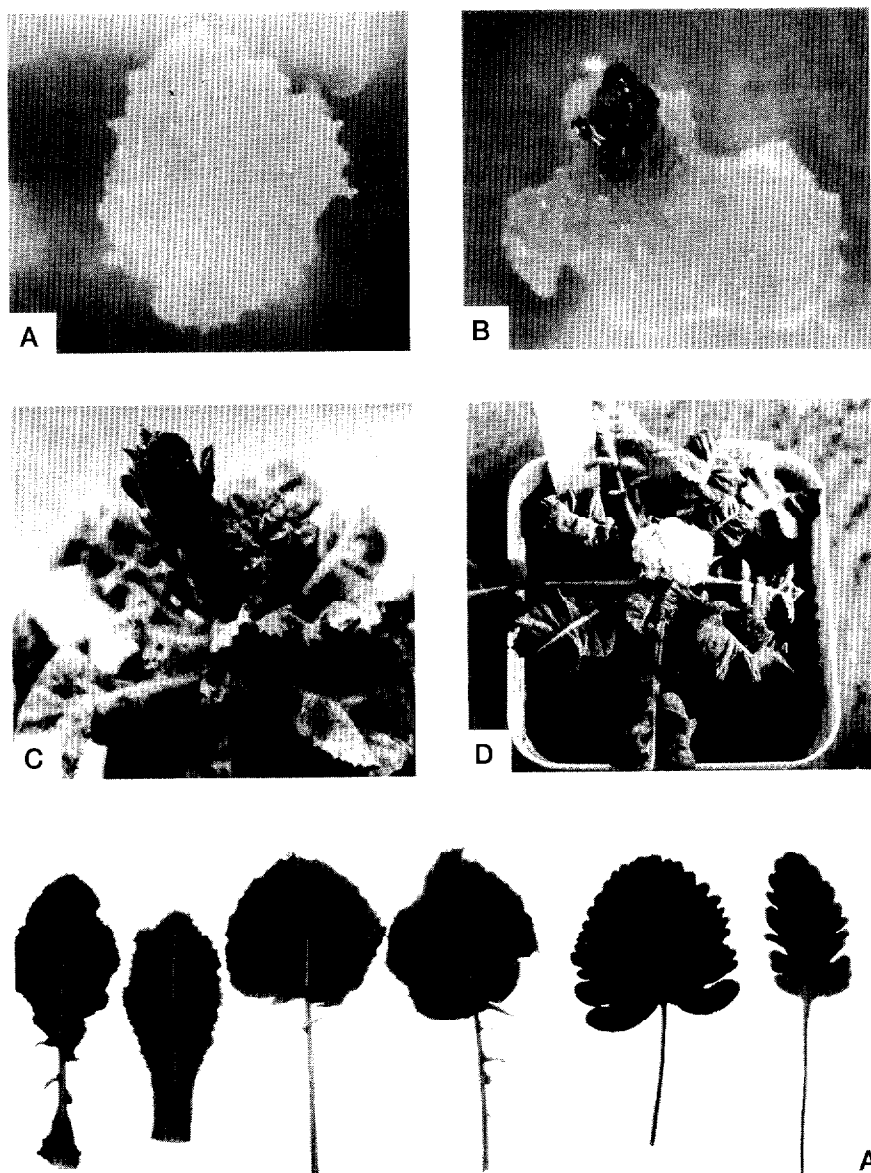


Fig. 1. Appearance of parents (cauliflower, *Brassica oleracea* var. *botrytis*; horseradish, *Armoracia rusticana*) and protoplast fusion regenerants of somatic hybrids: *a*) White, non-proliferating callus. *b*) A callus with anthocyanescent meristematic areas. *c*) Hybrid (aneuploid). Note the virescent transformation of cauliflower type head. *d*) Hybrid (aneuploid) regenerant No. 28. *e*) Two petiolate leaves characteristic for "Armobrassica" hybrid (couple in the middle) compared with two characteristic cauliflower leaves (couple from left) and characteristic leaves of horseradish (couple from right).

colour of leaves was green to greyish-green with persisting antocyanescent areas on leaves. Numerous morphological types were observed within the population of regenerated plants: the plants varied from subtle to robust, the shape of leaves was round to oblong with variable shapes of leaf margins, from smooth to rugose leaf blade. Small petiolar wings characteristic for cauliflower were smaller or even absent in some plants, the petioles resembled those of juvenile horseradish leaves (Fig. 1e).

The development of flowers was delayed and irregular. Virescent or anthocyanescent leaves and leafy buds proliferated in some parts of heads (Fig. 1c). The flowers were sterile with deformed pistils and anthers, so no seeds have been obtained as yet.

Table 3. Chromosome numbers in plants regenerated after (*A. rusticana* + *B. oleracea*) protoplast fusion.

Number of plants evaluated	Number of plants with chromosome number*				
	2n	4n	8n	aneuploid**	mixoploid (2n, 4n)
89	8	62	2	14	3

\*chromosome numbers were obtained using root tip method (see Materials and methods). Five to ten mitoses were evaluated in each plant; \*\* - three types of aneuploids were detected: with 34, 35 and 37 chromosomes, respectively.

Table 4. Chromosome numbers in 14 aneuploid hybrid (*A. rusticana* + *B. oleracea*) plants (regenerants marked with the same index letter were derived from the same microcallus).

Chromosome number*	Hybrid plant number/microcallus number
34	15/4, 21/30, 28/28, 71/22, 78/40, 80/9
35	74/22, 79/40, 81/30
37	53/7, 54/7, 55/7, 70/26, 83/26

\* Five to ten mitoses were evaluated in each plant.

**Cytology and ploidy variation in hybrids:** Preliminary chromosome analysis of regenerants after fusion of *B. oleracea* + *A. rusticana* protoplasts revealed a majority of plants with 36 chromosomes (which corresponds to tetraploid constitution of *B. oleracea* chromosomes), but aneuploid and mixoploid plants were also found (Table 3). The chromosomal constitution of 14 aneuploid hybrid plants was reconfirmed using the root tip meristems (Table 4). Occasionally, variation in the ploidy level among cells within the same meristem was also observed (Fig. 2a) including small chromosomal fragments (Fig. 2b). The only chromosome which could be identified was the chromosome No. 6 of *B. oleracea* genome (Olin-Fatih and Heneen 1992), a submetacentric one with a clearly distinguishable satellite. This was observed in hybrids Nos. 21 and 28 and present pairwise in the cells with 34 chromosomes (Fig. 2b).

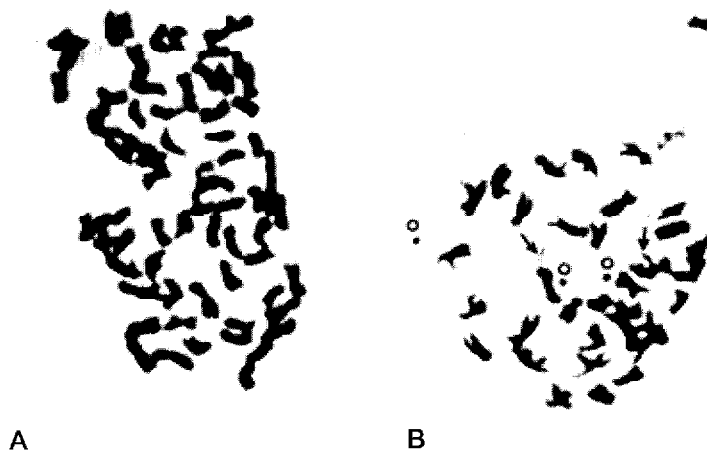


Fig. 2. Metaphases of hybrid plants: a) Chromosomes in metaphase plate of hybrid No. 28 possessing 68 chromosomes. b) Early metaphase plate of the same hybrid, 34 chromosomes. Note the presence of chromosome fragments (*arrowheads*). Chromosomes No. 6 from *B. oleracea* genome can be identified having distinguishable satellites (*arrowed*).

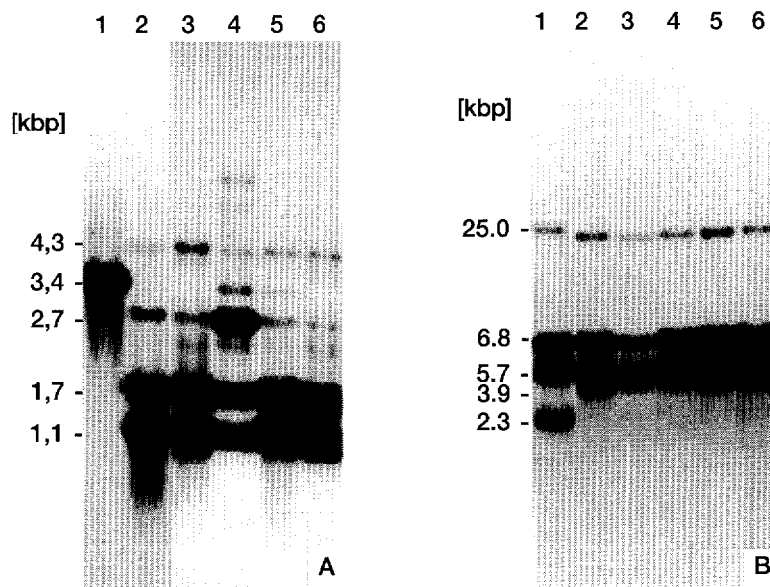


Fig. 3. Autoradiograms of DNA/DNA hybridizations: a) Hybridization of parental and hybrid genomic DNAs with 25S-rDNA probe. Lanes: 1. *A. rusticana* DNA, 2. *B. oleracea* DNA. 3 to 6, DNAs from hybrids: 70, 28, 21 and 15, respectively. b) The same DNAs hybridized with chloroplast DNA probe pTB18. Molecular masses of prominent bands are indicated in kbp.

**DNA hybridization with 25S-rDNA:** In *B. oleracea*, four distinct bands were detected (4.3, 2.7, 1.7, and 1.1 kbp) while in *A. rusticana* only a single band of 3.4 kbp in size was found (Fig. 3a). This *A. rusticana*-specific band was also present in the genomic DNAs of hybrid clones Nos. 28 and 21 (Fig. 3a, lanes 4 and 5). However, the intensity of this *A. rusticana*-specific hybridization signal was lower when compared to control *A. rusticana* DNA. In eight remaining DNA samples isolated from putative hybrids only *B. oleracea*-specific hybridization patterns were found (*cf.* lanes 3 and 6 in Fig. 3a).

**DNA hybridization with pTB18 cpDNA probe:** The same genomic DNAs used in the 25S-rDNA hybridization experiment were probed with pTB18 chloroplast DNA probe. *B. oleracea* and *A. rusticana* DNAs yielded five distinct hybridization signals; four of them were of a similar size in these two species. Their hybridization patterns differed in the shortest band possessing the size of 3.9 kbp in *B. oleracea* and 2.3 kbp in *A. rusticana* (Fig. 3b). In all the DNA samples isolated from the hybrid clones only the *B. oleracea*-specific pattern was detected, which does not confirm the hybrid state of cytoplasm genome.

## Discussion

*Armoracia rusticana* and *Brassica oleracea* are distantly related plant species. They belong to different sub-tribae of *Cruciferae* family showing different basic chromosome numbers, ploidy levels (*A. rusticana*  $x = 8$ ,  $2n = 4x = 32$ , *B. oleracea*  $x = 9$ ,  $2n = 2x = 18$ ), and geographic origin (Hedge 1976, Prakash and Hinata 1980). Our previous attempts with sexual crosses of two *Armoracia* species (*A. rusticana* and *A. sisymbrioides*) with *B. oleracea* failed even when the ovary culture was used to rescue resulting embryos (unpublished results).

Composite genomes are frequent within *Cruciferae* crops (U 1935, Warwick and Black 1991) and their nature can be verified by experimental reconstruction through sexual and/or parasexual hybridizations (for review see Glimelius *et al.* 1991). Nevertheless, the increasing taxonomic distance contributes to growing frequency of asymmetric somatic hybrids (Prakash and Hinata 1980, Perl *et al.* 1991). Uneven distribution of both nuclear and cytoplasmic elements seems to be dependent from both type and cell cycle of protoplasts used for fusion, so that the results of different fusions may be very variable (Sundberg *et al.* 1991). The fusion method of construction of somatic hybrids used in this work was similar to those of Toriyama *et al.* (1987) and Kirti *et al.* (1992) with "*Brassicomoricaandia*" regarding the use of mesophyll and hypocotyl protoplasts as well as the selective ability of culture media.

The plants derived from protoplast fusions have usually an intermediate phenotype, resembling both parental species in various traits (Terada *et al.* 1987, Kameya *et al.* 1989). In our case, the appearance of putative hybrid plants resembled the *B. oleracea* parent, but some morphological traits revealed the presence of horseradish germplasm. The most striking morphological features were the early

anthocyanescence of regenerating shoots and the morphology of cauliflower curds (Figs. 1c,d).

It is well documented that in plant somatic hybrids (mainly in those derived from distantly related species) aneuploidy, chromosome rearrangements, and specific chromosome elimination of one parent frequently occur (Hoffman and Adachi 1981, Wolters *et al.* 1994). These phenomena have also been observed in this work, namely aneuploidy, mixoploidy, and chromosome fragments. Chromosomal imbalances are probably due to the interactions of parental genotypes and to somaclonal variation (Scowcroft 1985) since the plants passed the *in vitro* regeneration. The karyological stability of hybrids seems also dependent on both the cell type and the stage of the cell cycle of protoplasts used for fusion, so the results of different fusion experiments may be highly variable (Sundberg *et al.* 1991).

None of the plants tested possessed the sum of parental chromosome sets. In the majority of metaphases of hybrid plants the pair of typical submetacentric chromosomes with distinguishable satellites can be identified as the chromosome No. 6 of *B. oleracea* (Olin-Faith and Heneen 1992). The presence of only two copies of this particular chromosome in hybrid karyotypes is an indirect evidence that only one *B. oleracea* genome was involved in the original fusion and thus the hybrids are not *Brassica-Brassica* fusion products.

Southern hybridizations using nuclear and organelle DNA probes are widely used to evaluate the hybrid character of plants (*e.g.*, Chevre *et al.* 1994). We used two DNA probes to follow the nuclear as well as the cytoplasmic component of the putative hybrids. In hybrids Nos. 21 and 28, the *A. rusticana*-specific, 25S-rDNA restriction fragment was detected in their genomic DNAs. However, the relatively low intensity of this hybridization signal indicates that the hybrids were asymmetric with only a small proportion of *A. rusticana* genome, probably due to specific chromosome elimination. In the majority of plants tested, the *A. rusticana*-specific 25S rDNA hybridization pattern was not detected. Moreover, all the investigated clones (including Nos. 21 and 28) had only *B. oleracea*-specific hybridization pattern for the pTB18 chloroplast DNA probe. This may indicate the chloroplast elimination, a phenomenon often observed in plant somatic hybrids (Landgren and Glimelius 1990, Sundberg and Glimelius 1991).

From the data summarized here it can be stated that introgressive parasexual hybridization between *Brassica* and *Armoracia* genomes could be functional and may lead to production of asymmetric hybrids, albeit containing only small fragments of the *Armoracia* genome.

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