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"Arabidobrassica": Chromosomal recombination and morphogenesis in asymmetric intergeneric hybrid cells

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Abstract. A somatic hybrid cell line, cloned from an individual protoplast-fusion product between *Arabidopsis thaliana* and *Brassica campestris*, gave rise to formation of numerous plants differing drastically in morphology. Analysis of these various regenerants, all of which originated from one and the same heterokaryon derived from the fusion of two cells, shows the unspecific elimination of chromosomes of both parental species during the callus growth phase. Whereas the parental cells have so far not been successfully regenerated into plants, several of their different asymmetric hybrids are capable of morphogenesis. Furthermore, chromosomal analysis indicates extensive recombination. Most of the plants are predominantly morphologically regular. Abnormalities are mostly limited to the flowers which tend to undergo phyllody. The results demonstrate that remote somatic hybridization may have applications although true amphidiploids may not be obtainable. The transfer of small units of genetic material between distantly related species by protoplast fusion seems to be a more realistic approach than the combination of complete, highly diverse genomes.

Key words: *Arabidopsis* – *Brassica* – Chromosome elimination – Protoplast fusion – Somatic hybrid.

Introduction

We have recently described the regeneration of a symmetric and an asymmetric hybrid plant from two different callus lines obtained by protoplast fusion between *Arabidopsis thaliana* and *Brassica campestris* (Gleba and Hoffmann 1979, 1980). The symmetric "Arabidobrassica" did not show indications of chromosomal elimination and is probably a stable, true allopolyploid intertribal hybrid (this is why it is termed symmetric, in contrast to asymmetric hybrids, in which parts of one or both parental genomes are eliminated). In the asymmetric "Arabidobrassica", none of the *Brassica* marker chromosomes, i.e., chromosomes which can be clearly distinguished from those of *Arabidopsis*, could be identified but we found conclusive evidence that *Brassica* genes were active in this regenerant. Examples were trichome structure, petal color, the complementation of the *Arabidopsis* nuclear albino mutation (Gleba and Hoffmann 1980), the proportion of condensed chromatin (Nagl and Hoffmann 1980), and the *Brassica* plastidome (Komarnitsky and Gleba, in press). The fact that two different genomes which are each capable of ordered morphogenesis can originate from combination of the same species after protoplast fusion raises the question whether additional genomes are also capable of regenration and whether the degree of asymmetry can be influenced experimentally. If so, the method of protoplast fusion and chromosomal elimination would therefore open up the possibility to transfer parts of the genome or even single chromosomes from one species to another. Furthermore, indications are available that reconstruction of chromosomes may occur in cells of remote hybrids (Gleba and Hoffmann 1978) and this would permit the transfer of even parts of single chromosomes between species by means of protoplast fusion.

Materials and methods

Hybrid cell lines of *Arabidopsis thaliana* L. (×) *Brassica campestris*[1] L. (Steinacher Winterrübsen) were obtained by mechanical isolation and culturing of individual protoplast-fusion products (Gleba and Hoffmann 1978). Leaves from aseptically grown plants of *Brassica* were used as source of mesophyll cell protoplasts ($2n = 2x = 20$) and an *Arabidopsis* culture as that of callus cells protoplasts (predominantly octoploid, $2n = 8x = 40$). Callus tissues were initiated from chlorophyll-deficient seedlings of a recessive nuclear albino mutant. The *Arabidopsis* cell line did not exhibit any morphogenetic

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1 A somatic hybrid between species A and species B should be designated as A (X) B to distinguish it from the sexual hybrid, A X B, and the graft chimaera, A + B (for details see Hoffmann, in press)

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activity during the last two years of culture whereas freshly isolated callus cultures derived from mesophyll protoplasts of *Brassica campestris* occasionally produced roots but did not produce any shoots (Schenck and Hoffmann 1979). The hybrid cell lines were cultured in the dark at 26° C on solid B_5 medium (Gamborg et al. 1968) modified to contain 1 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (Serva, Heidelberg, FRG), 0.2 mg l^{-1} of 6-benzylaminopurine (Fluka, Buchs, Switzerland), and 0.2 mg l^{-1} of 1-naphthaleneacetic acid (Merck, Darmstadt, FRG). Medium B_5 with 1.5 mg l^{-1} of 6-benzylaminopurine as the sole phytohormone was used for shoot-bud induction under daily illumination for 16 h with 6,000 lx from Osram L-40-Natura lamps. Regenerating plantlets were transferred to the medium of Linsmaier and Skoog (1965), supplemented with 0.1 mg l^{-1} 1-naphthaleneacetic acid and 3 g l^{-1} active carbon (Merck 2186, Darmstadt, FRG) for further growth and rooting.

Analyses of chromosomes and of esterase isozymes were conducted as described earlier (Gleba and Hoffmann 1978). Densitograms of gels were made using a Quick Scan Densitometer (Desaga, Heidelberg, FRG) by scanning at 523 nm.

Results

Four hybrid cell lines with 60 chromosomes which had not previously shown chromosomal elimination and shoot regeneration were transferred to the callus medium and placed in the dark. Every four weeks about half of the cultured material was transferred back to regeneration medium. The results reported here were obtained with one cell line, ab 16. Another cell line produced one regeneration site but the developing plantlet could not be maintained.

After four weeks of growth on the callus medium in the dark, changes in esterase isozyme pattern of hybrid cell line ab 16 were visible. Densitograms from randomly taken callus pieces are shown in Fig. 1. A comparison of the three fast moving bands which have been used for the identification of hybrid lines

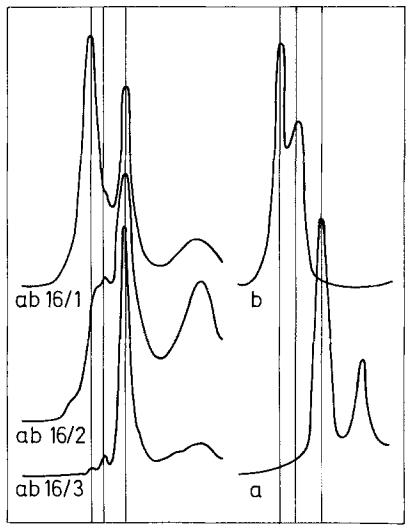

Fig. 1. Densitograms of the fast-moving esterase isozyme bands which have been used for the identification of hybrid cell lines of *Arabidopsis* (×) *Brassica*. In non-chromosome-eliminating cells, the two *Brassica* (*b*) and the one *Arabidopsis* (*a*) peaks were shown to be expressed simultaneously (for illustration see Gleba and Hoffmann 1978). Randomly taken callus pieces of the chromosome-eliminating line ab 16 (ab 16/1; ab 16/2; ab 16/3) display the loss of one of the distinctive *Brassica*-peaks, or of both

(Gleba and Hoffmann 1978) indicates the onset of the elimination of chromosomes. In the next transfer, eight weeks after the start of the experiment, formation of a plant was observed the first time. This first regenerant obtained is shown in Fig. 2A and Fig. 2B presents the second regenerant which was obtained four weeks later. Although the two shoots originated from the same fusion product, their morphologies

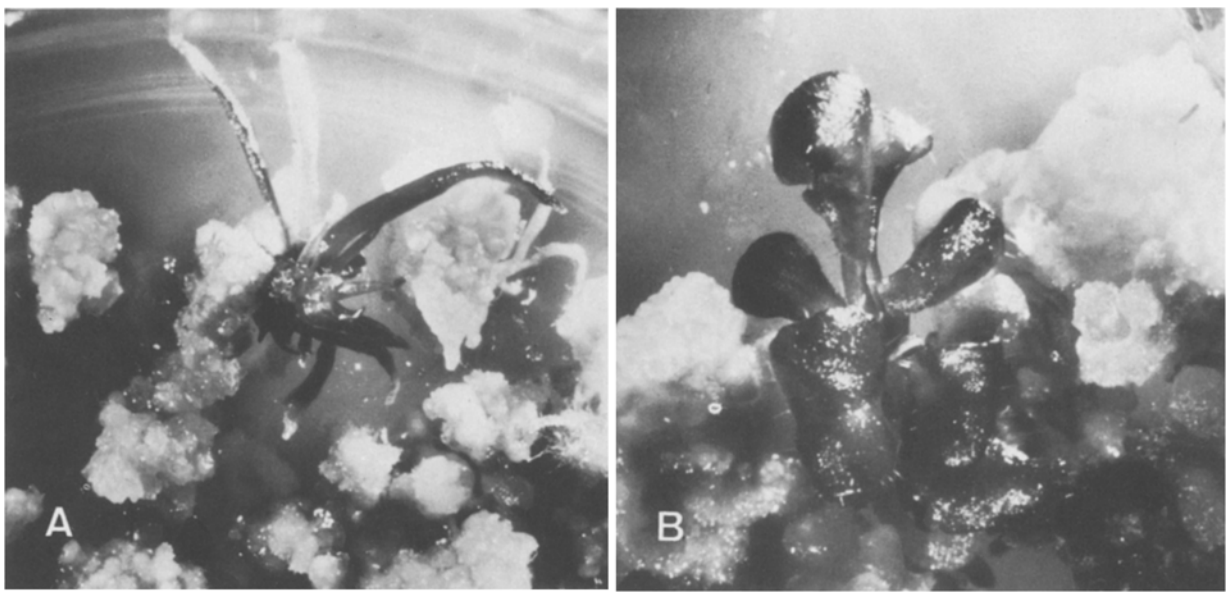

Fig. 2A, B. First (**A**) and second (**B**) regenerated shoot of "Arabidobrassica" obtained from cell line ab16

Fig. 3A–D. Selection of phenotypes regenerated from the hybrid cell line ab 16 (plantlets are cultured in plastic containers with a height of 11 cm): **A** *Arabidopsis*-like plantlet; **B** divergent plantlet; **C** *Brassica*-like plantlet; **D** intermediate plantlet

were markedly different. Thereafter, the morphogenetic activity of this cell line increased dramatically. Several regeneration sites were regularly formed in most explants. Their production was under observation for up to twelve months and 305 regenerants were isolated. This number reflects the capacity of our laboratory rather than the regeneration potential of this cell line. The morphological diversity between these isolates is extreme, ranging from predominantly *Arabidopsis*-like plants over intermediate ones to more *Brassica*-like plants, plus types that cannot be assigned to any of these three. Some of the phenotypes are illustrated in Figs. 3 and 4. The distribution of plant types observed in plantlets at comparable developmental stages from 69 different regeneration sites (the number of comparable plants available in our culture room on one randomly taken day) is shown in Table 1. The classification was arbitrarily based on two characters, the overall appearance and the trichome structure, in order to give a vague idea of the segregation obtained. *A. thaliana* leaves have trichomes with three branches, while *B. campestris* leaves have only simple nonbranching trichomes (Fig. 4, for details see illustrations in Gleba and Hoffmann 1979, 1980). Intermediates are those with hairs with two branches and all others (i.e., without trichomes, with a mixed population of trichomes or deformed trichomes) are classified as divergents. A rigorous analysis of the variability is not feasible due to the tremendous complexity of the material. It should be stressed once more that all the regenerants go back to a single hybrid cell.

Ten plants, derived from different regeneration sites, were selected for chromosomal analyses, although chromosome counting and identification is difficult and not always unequivocal in this material (Gleba and Hoffmann 1978). All plants had chromosome numbers between 35 and 45. Chromosomes of both parents were identifiable in all cases. Chromosome numbers in different organs of the same plant and in vegetatively propagated plants from the same regeneration site were stable within ± 1, indicating that organization into tissue seems to result in keeping the genome balanced. However, structurally changed chromosomes (ring chromosomes, multiconstrictional chromosomes) were visible in all metaphases and in six regenerants very extensive chromosomal recombination was observed (Fig. 5). A comparable accumulation of recombination figures has not been observed in the parental callus cultures and we have never seen anything comparable in any other tissue cultures.

The phenotypes of most of the regenerants remained stable during vegetative propagation, supporting the cytological evidence for the genome being balanced in organized tissue. However, nine lines were found to exhibit segregation (and involuntarily selection on the part of the person making the transfer) and other three showed a drift towards a different

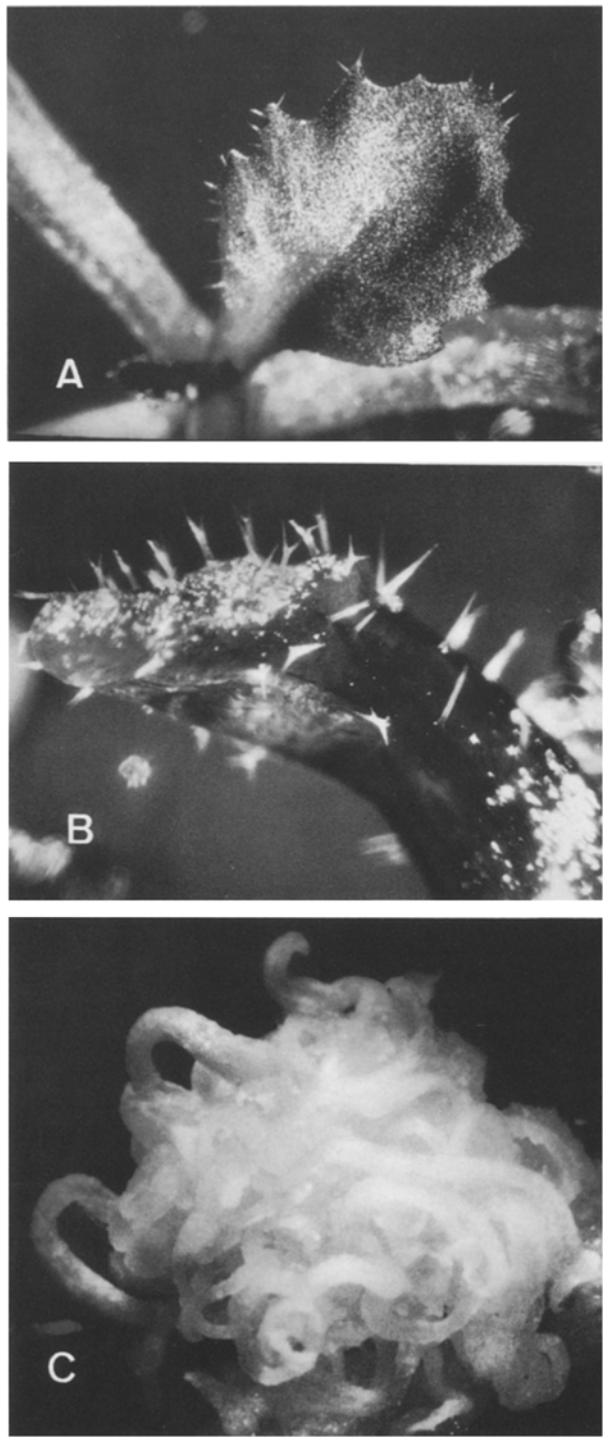

Fig. 4A–C. Leaves and leaf-like structures of regenerants from the hybrid cell line ab 16: **A** *Brassica*-like leaf with typical unbranched trichomes; **B** teratoma leaf-like structure with predominantly intermediate trichomes; **C** albino segregant without trichomes

Table 1. Distribution of *Arabidopsis*-like (A), *Brassica*-like (B), intermediate (I), and divergent (D) plants between 69 regenerants from a single "Arabidobrassica"-hybrid cell. In (a) the classification was made by estimating the overall appearance and in (b) by using trichome structure

Character	Regenerants			
	A	B	I	D
a	14	6	37	12
b	2	23	4	40

phenotype. In two cases, this phenotypic drift proceeded from teratoma- (Fig. 4B) to callus-like growth. Very recently, a secondary callus, which originated spontaneously from the basis of an unrooted plantlet, produced albino shoots (Fig. 4C) and is the first white tissue we have obtained from "Arabidobrassica" cells (the parental *Arabidopsis* cells were from chlorophyll-deficient seedlings of a recessive nuclear albino mutant).

From three regeneration sites only highly abnormal shoot- and leaf-like structures were obtained, similar to those obtained in the first asymmetric "Arabidobrassica"-hybrid (Gleba and Hoffmann 1979). Secondary callus, spontaneously formed from these teratomata, grew without exogenous hormones. This autotrophy may be the consequence of a chromosomal situation which is comparable to genetic tumors. The majority of the regenerants observed were morphologically essentially normal. A common problem was difficulty in inducing root formation, in contrast to rooting in callus which is easy to obtain (Gleba et al. 1978). Only ten shoots obtained from seven different regeneration sites have been successfully rooted. Morphologically perfect flowers were found (Fig. 6A), with the classical formula of the Crucifer family. Placentae with ovules and pollen were formed (Fig. 6C). However, nothing can be said about their functionality. Self fertilization has so far not resulted in production of seeds and crosses between different regenerants or backcrosses to the original parent species have not been performed.

Phyllody of an anther is shown in Fig. 6D as an intermediate stage between a perfect Cruciferous flower and the double flowers without sexual organs (Fig. 6B) which were also obtained earlier (Gleba and Hoffmann 1980). Anthers with functional stomata have sometimes also been found in nature (Kenda 1952), but the case in question (stomata in connection with a reduced archesporium, Fig. 6D) clearly represents the transformation of reproductive into vegetative tissue. Obvious correlations between phenotype and genotype could not be recognized, in that *Brassica*-like hybrid plants did not always contain more *Brassica* chromosomes and *Arabidopsis*-like hybrid plants did not always contain more *Arabidopsis* chromosomes than chromosomes of the respective other plant species.

Discussion

Three aspects of our results are of major interest: 1. An intertribal hybrid cell line from the Crucifer

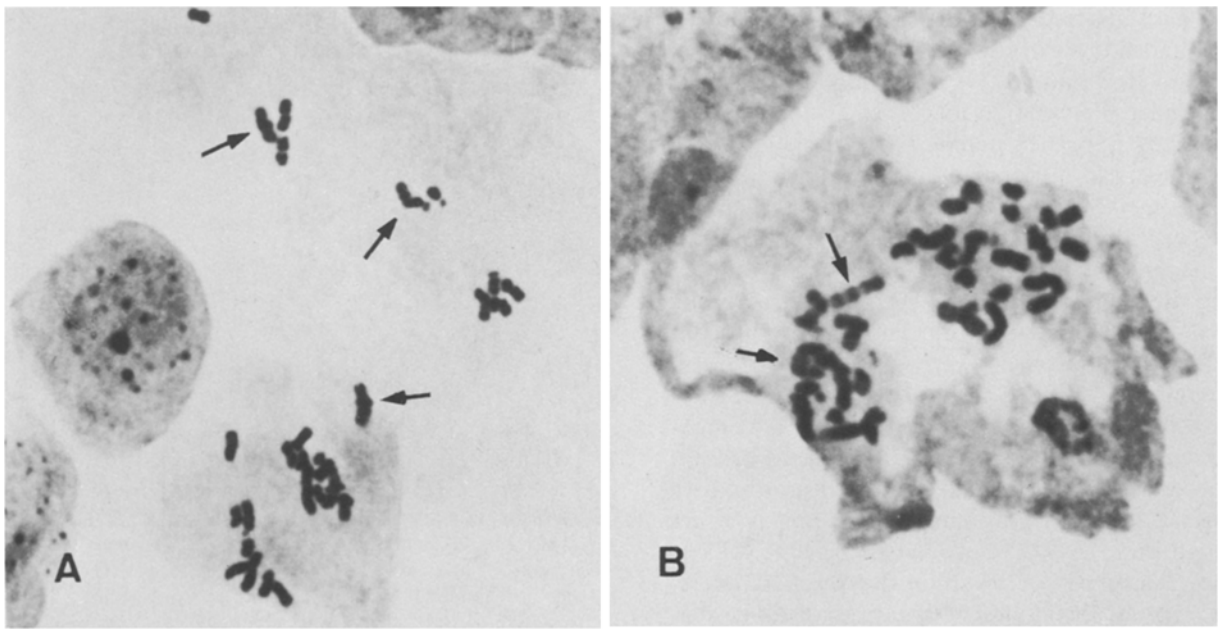

Fig. 5A, B. Metaphase chromosomes in stigma cells from two different plantlets of "Arabidobrassica" showing structurally changed chromosomes (indicated by *arrows*)

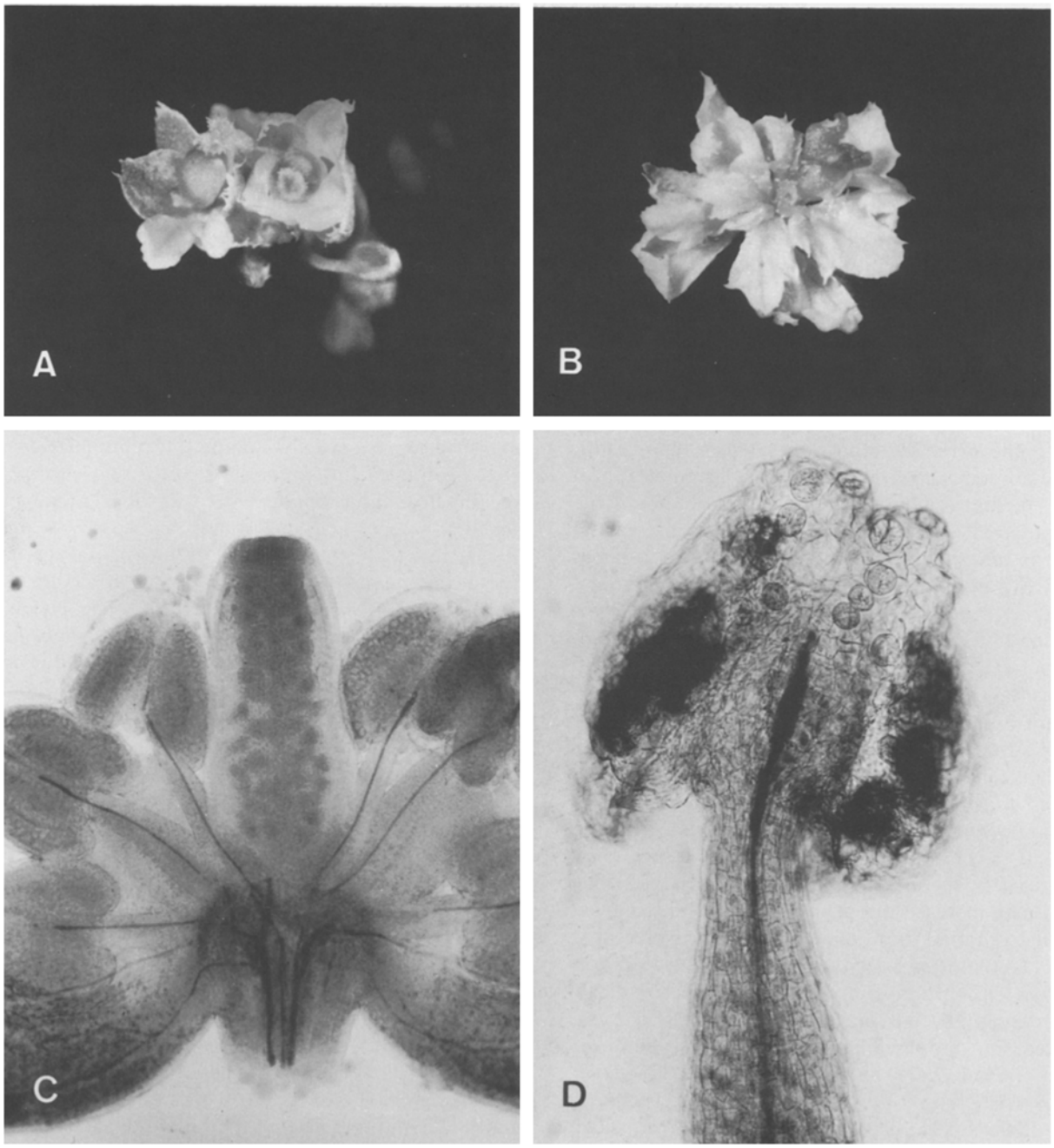

Fig. 6A–D. Flower types of plants regenerated by "Arabidobrassica" cell line ab 16: **A** morphologically regular Cruciferous flower; **B** double flower with completely reduced sexual organs; micropreparations showing (**C**) anthers with pollen grains and an ovary with ovules, and (**D**) an anther with stomata and reduced pollen sacs

family is characterized by mass regeneration of plantlets. It has not yet been possible, in most cases, to obtain consistent shoot formation from isolated protoplasts of non-Solanaceous plants and this is especially true of the *Brassica*-species (Hoffmann et al. 1980; Kohlenbach et al., in press). 2. The interplay of genome fusion and chromosomal elimination has led to the formation of several different genomes which all can be traced back to a single hybrid cell and which can be stabilized by organogenesis. 3. As a consequence of hybridization, intrachromosomal recombination occurs in the hybrid cells and strong indications are available that this recombination may involve chromosomes of the two parents, i.e., plants belonging to different tribes of a family.

Maliga et al. (1977) have reported restoration of the morphogenetic potential of plant cells by somatic hybridization in *Nicotiana* and have suggested genetic

complementation and regulatory changes by the interaction of different genomes as possible explanations. In our case, the *Arabidopsis* parental cell line may have irreversibly lost its morphogenetic potential during prolonged culture (since 1975) and because of genome mutation from 2X to 8X. The failure to obtain plant regeneration from *Brassica campestris* protoplasts is undoubtedly because the proper culture conditions have not yet been found. The hybrid "Arabidobrassica" cells produced cell lines which could be induced both to single and multiple shoot formation, as well as lines in which shoot formation could not be induced under the conditions used. However, these differences in the morphogenetic potential are explainable without recourse to elaborate genetic models, on the basis of technical reasons. Slight differences in the early handling of the lines may result in large differences in the reaction to a given morphogenetic medium. The genetic probability should be the same in all cases. In this respect, it is interesting to bear in mind that all hybrid cells that were isolated already expressed hybrid vigour at the very beginning of their existence. Their first division had occurred by the end of the first day of culture and the second division by the third day (Gleba and Hoffmann 1978), whereas the parental protoplasts in the same medium were not observed to divide before the third day and did not sustain continued cell division.

Genetic instability after protoplast fusion has been reported in hybrid cell lines of distantly related species (Kao 1977) and in hybrid plants between *Nicotiana* species (Maliga et al. 1978). It may be argued that an increase in the evolutionary distance between the species may increase the probability of obtaining segregating genomes, but such a suggestion is presently without factual foundation. In the case of *Arabidopsis* and *Brassica*, chromosomal elimination is obviously not a necessity because a symmetric hybrid has been obtained. On the other hand, chromosomal elimination can be induced as an unspecific process. It seems to take place mainly during the unorganized growth phase of the hybrid cells. Organ formation can lead to a complete or gradual stabilization of the genome that had been subject to chromosome elimination (or vice versa), presupposing that the genome is capable of directing morphogenesis. It seems possible that such chromosome-eliminating cells fluctuate in their capacity for morphogenesis.

Indications for intergeneric intrachromosomal recombination has been reported by Dudits et al. (1979) in plants obtained after fusion of protoplasts from *Daucus carota* and *Aegopodium podagraria*. In our material, chromosomal recombination rarely occurs in non-chromosome-eliminating hybrid cell lines (Gleba and Hoffmann 1978). The dramatic increase of this process seems to be somehow linked to the phenomenon of chromosomal elimination. Protoplast fusion followed by chromosomal elimination, and probably also by chromosomal recombination, thus can lead to the transfer of units of genetic material smaller than complete chromosomes between plant species. In addition to the true allopolyploid hybrid, such asymmetric hybrids may be very useful, but they may be especially valuable where allopolyploidy is highly unstable. A similar approach has been already realized in *Triticum* by plant breeders, utilizing the meiotic instability of a hybrid genome, although in this case the addition, substitution and translocation lines of wheat started with a sexual cross (Zeller and Fischbeck 1974). There is however no obvious reason why obtaining such modified, fertile species should be restricted to sexually compatible combinations. Thus, protoplast fusion should make a conventional breeding technique applicable to a wider range of species.

The results on mitotic segregation of a somatic hybrid genome reported here and similar observations in *Datura* (×) *Atropa* parasexual hybrid tissue (Krumbiegel and Schieder, in press) should however not be used as an argument against attempts at also producing new fertile allopolyploid species by protoplast fusion. By serendipitous bypassing sexual incompatibility nature has demonstrated that amphidiploidy also works in hybrids between species which can normally not be sexually crossed. One such example is amphidiploid rape seed (*Brassica napus*, 2n=38), and attempts have been undertaken to copy the natural process by protoplast fusion between *Brassica oleracea* (2n=18) and *Brassica campestris* (2n=20) (Hoffmann et al. 1980). There is again no plausible reason why stable amphidiploidy between sexually incompatible plants should be restricted to the few randomly naturally occurring examples. Furthermore, stability of the genome of somatic hybrids between sexually incompatible species has already been demonstrated in *Datura* (Schieder 1978, 1980), potato (×) tomato (Melchers et al. 1978) and symmetric "Arabidobrassica" (Gleba and Hoffmann 1980).

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