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# Asymmetric somatic hybrids of *Brassica*: partial transfer of *B. campestris* genome into *B. oleracea* by cell fusion

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**Summary.** To examine the possibility of producing asymmetric somatic hybrids of *Brassica* having a complete genome of one species and a part of the other, we fused inactivated *B. oleracea* protoplasts with X-irradiated *B. campestris* protoplasts. The plants obtained were studied with regard to their morphology, isozymes and chromosomes. The morphology of the hybrids was similar to *B. oleracea* in 9 out of 22 hybrids studied and the rest showed the intermediate phenotype of the parents. Analysis of three isozymes, leucine aminopeptidase, acid phosphatase and esterase indicated that ten hybrids lost *B. campestris*-specific bands in one or more of the three isozymes examined. The chromosome analysis showed that 90% of the hybrids were aneuploids. In addition, abnormal chromosomes were often found in root tip cells. These results suggested that the hybrids obtained were asymmetric in nature and resulted from elimination of *B. campestris* chromosomes by X-ray irradiation.

**Key words:** *Brassica* – Protoplast fusion – Asymmetric hybrid

## Introduction

Protoplast fusion provides a method for combining genomes of distantly related species (Schieder and Vasil 1980; Schieder 1982). However, various levels of genetic incompatibilities became evident in somatic hybrids of distant species (Kao 1977; Binding and Nehls 1978; Melchers et al. 1978; Gleba and Hoffmann 1980; Harms 1983; Terada et al. 1987 a). These genetic incompatibilities generally result in the failure to regenerate the fertile plants that are required for breeding or genetic studies. To overcome this problem, transfer of only desirable characters from diverse genomes by asymmetric somatic hybridization has been proposed (for review, see Dudits and Praznovszky 1985).

Dudits et al. (1980) first described the production of a carrot + parsley hybrid by irradiating parsley protoplasts by X-ray. In this instance, the chlorophyll deficiency of carrot was used as a selection marker to obtain hybrids. Similar studies on production of asymmetric hybrids by combining cell mutants and X- or γ-ray irradiation of donor protoplasts were described in *Nicotiana tabacum* + *Physalis minima*, *N. tabacum* + *Datura innoxia* (Gupta et al. 1982), *N. glauca* + *N. langsdorffii* (Itoh and Futsuhara 1983), *D. innoxia* + *P. minima* (Gupta et al. 1984), *N. tabacum* + *Hordeum vulgaris* (Somers et al. 1986), *Solanum tuberosum-phureja* + *S. pinnatisectum* (Sidorov et al. 1987) and *Hyoscyamus muticus* + *N. tabacum* (Imamura et al. 1987).

More recently, dominant selectable markers were used to obtain asymmetric somatic hybrids. Bates et al. (1987) employed kanamycin resistance as a selectable marker to obtain asymmetric *N. tabacum* + *N. plumbaginifolia* hybrids and Dudits et al. (1987) introduced 5-methyltryptophan resistance and methotrexate resistance of carrot into *N. tabacum* and obtained fertile asymmetric hybrids.

Although somatic hybrids and cybrids of *Brassica* species have been described (Schenk and Röbbelen 1982; Pelletier et al. 1983), no instances of asymmetric hybrids containing a complete genome of one species and a part of the genome of the other have been reported. This could be partly due to the lack of mutants selectable at the cellular level in these species.

In order to examine the possibility of producing asymmetric somatic hybrids in *Brassica*, we chose *B. ole-*

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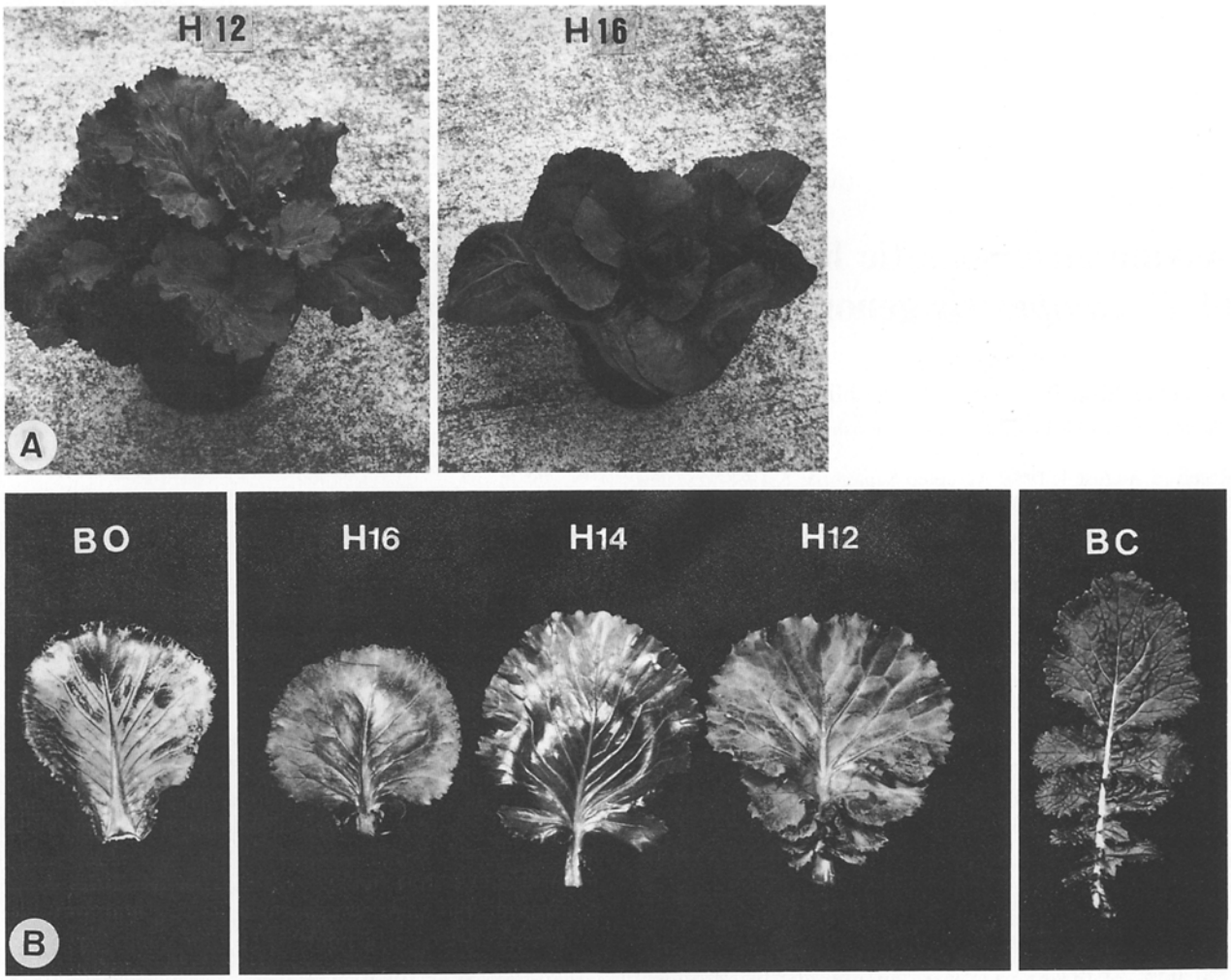


**Fig. 1 A and B.** Morphology of the hybrids. **A** Intermediate-type hybrid, H12 (left) and *B. oleracea*-type hybrid H16 (right). **B** Leaf morphology of the parents, *B. oleracea* var. capitata cv. Nakawase rubi (BO) and *B. campestris* var. rapa cv. 77b (BC) and three hybrids, *B. oleracea* type (H16 left) and Intermediate-type (H14 middle, H12 right)

*racea* and *B. campestris* as fusion parents because we have previously developed an efficient method of production of somatic hybrids between these two species (Terada et al. 1987b). By fusing inactivated *B. oleracea* protoplasts and X-irradiated *B. campestris* protoplasts, we obtained a number of hybrids. The asymmetric nature of the hybrids was suggested by their morphology, chromosome analysis and isozyme banding pattern. Also, a relationship between X-ray dose and the degree of asymmetry in the chromosome constitution of the hybrids was investigated.

## Materials and methods

*Plant materials*

*B. oleracea* var. capitata cv. Nakawase rubi, Shikidori, Yonmaru and Shutoku (cabbage) were used as one parent and *B. campestris*, var. rapa cv. 77b, var. pekinensis cv. CR strong and Yokozuna (chinese cabbage) were used as another parent.

*Protoplast isolation, irradiation, fusion and culture*

Isolation of hypocotyl protoplasts of *B. oleracea* and mesophyll protoplasts of *B. campestris* was as described previously (Terada et al. 1987b). *B. oleracea* protoplasts were treated with 20 m*M* iodoacetamide (IOA) for 15 min at 4°C. *B. campestris* protoplasts ($2.5 \times 10^5$/ml) in the W5 solution (Menczel and Wolfe 1984) were irradiated by X-ray in a range of 10–80 kR (Ohmic, 40 kV, 4 mA, 1 kR/min). Protoplasts were then mixed at a 1:1 ratio with a density of $3–4 \times 10^6$/ml and fused according to Menczel and Wolfe (1984).

Fused protoplasts were cultured in KM8p liquid medium (Kao and Michayluk 1975) at a density of $10^6$ protoplasts/ml. After 1 week, cell density was reduced to one-half by the addition of fresh medium. After 3 weeks of culture in the liquid medium, the protoplasts were placed on solid MS-NN medium containing MS inorganic salts (Murashige and Skoog 1962), vitamins of NN67 (Nitsch and Nitsch 1967), 0.03 *M* sucrose, 0.2 *M* mannitol, 1.0 mg/l 2,4-dichloroacetic acid, 0.3 mg/l kinetin and 0.25% agarose (type I, Sigma). To induce shoot regeneration, MS-NN medium supplemented with 0.015 *M* sucrose, 0.2 *M* mannitol, 0.1 mg/l indole acetic acid, 2.0 mg/l zeatin, 2.0 mg/l kinetin and 0.75% agarose was used. The medium for shoot

**Table 1.** Morphology, chromosome number and isozymes of somatic hybrids between *B. oleracea* and *B. campestris*

| *B. oleracea* | *B. campestris* | Plant no. | X-ray dose (kR) | Morphology | Isozymes | | | Chromosome no. | No. of *B. campestris* chromosomes eliminated |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | LAP | APS | EST | | |
| cv. 'Shikidori' | cv. 'CR strong' | H 1 | 10 | Int. | Int. | Int. | Int. | 54 | 2 |
| | | H 2 | | Int. | Int. | * | Int. | 74 | ** |
| | | H 3 | | Int. | Int. | Int. | Int. | 56 | 0 |
| | | H 4 | | Int. | Int. | Int. | Int. | 55 | 1 |
| | | H 5 | | B.O. | Int. | Int. | Int. | 34 | 4 |
| cv. 'Shutoku' | cv. 'Yokozuna' | H 6 | 20 | Int. | Int. | B.O. | Int. | 46 | 10 |
| cv. 'Shikidori' | cv. 'CR strong' | H 7 | 30 | Int. | Int. | Int. | Int. | 56 | 0 |
| | | H 8 | | Int. | Int. | B.O. | Int. | 45 | 11 |
| cv. 'Yonmaru' | cv. '77b' | H 9 | 30 | B.O. | B.O. | Int. | B.O. | 45 | 11 |
| | | H10 | | B.O. | B.O. | Int. | Int. | 42 | 14 |
| | | H11 | | Int. | Int. | Int. | Int. | 41 | 15 |
| cv. 'Nakawase rubi' | cv. '77b' | H12 | 60 | Int. | B.O. | Int. | Int. | 46 | 10 |
| | | H13 | | Int. | Int. | Int. | B.O. | 47 | 9 |
| | | H14 | | Int. | Int. | Int. | Int. | 43 | 13 |
| | | H15 | | B.O. | B.O. | B.O. | B.O. | 43 | 13 |
| | | H16 | | B.O. | Int. | Int. | B.O. | 47 | 9 |
| | | H17 | | Int. | Int. | Int. | Int. | 44 | 12 |
| | | H18 | | B.O. | Int. | Int. | Int. | 44 | 12 |
| cv. 'Nakawase rubi' | cv. 'CR strong' | H19 | 80 | B.O. | Int. | Int. | B.O. | 48, 30 | 8 |
| | | H20 | | B.O. | Int. | Int. | B.O. | 48 | 8 |
| | | H21 | | Int. | B.O. | Int. | Int. | 47 | 9 |
| cv. 'Shikidori' | cv. 'CR strong' | H22 | 80 | B.O. | Int. | Int. | Int. | 46 | 10 |

Int = Intermediate type
B.O. = *B. oleracea* type
* a not calculated
** b both *B. oleracea* and *B. campestris*-specific bands were missing

elongation and rooting was as described previously (Terada et al. 1987b). All the hybrid plants were grown in a growth chamber.

*Chromosome analysis*

Chromosome counts were made with root tip cells of regenerated plants according to the method previously described (Nishibayashi and Kaeriyama 1986).

*Isozyme analysis*

Leaf extracts of regenerated plants were subjected to polyacrylamide gel electrophoresis for esterase (EST) (Nakai 1976) and starch gel electrophoresis for leucine aminopeptidase (LAP) and acid phosphatase (APS) (Arús and Orton 1983).

## Results

*Cell fusion, selection and regeneration of hybrid plants*

Fusion frequency observed in a number of experiments was 5%–10%. High cell density ($> 1 \times 10^6$ protoplasts/ml) was required to obtain colonies, because inactivated parental cells inhibited the continuous division of fused cells by releasing toxic compounds such as phenolics. Therefore, the culture medium was diluted with fresh medium 1 week after fusion when the first division was initiated.

To eliminate *B. oleracea* regenerants, we employed 20 m*M* IOA instead of the 15 m*M* used in our previous study. We found that fusion frequency or viability of the fused cells was not affected by this increased IOA concentration. Based on the isozyme screening, 22 of 24 regenerated plants were found to be hybrids and these were studied further.

The frequency of plant regeneration was low in this experiment compared to that in the symmetric fusion experiment. To stimulate shoot formation, calluses were transferred to fresh regeneration medium several times at 1 month intervals.

*Analysis of somatic hybrids*

*1. Plant morphology*

A great deal of variation in the morphology was observed among the hybrids and this showed a marked contrast to the hybrids obtained without X-ray irradiation. The hybrids were classified into two groups according to their morphology: those similar to *B. oleracea* (B.O. type) and those showing the intermediate morphology between the parents (Int. type) (Fig. 1). Of the 22 hybrids studied, nine were B.O. type and 13 were Int. type (Table 1).

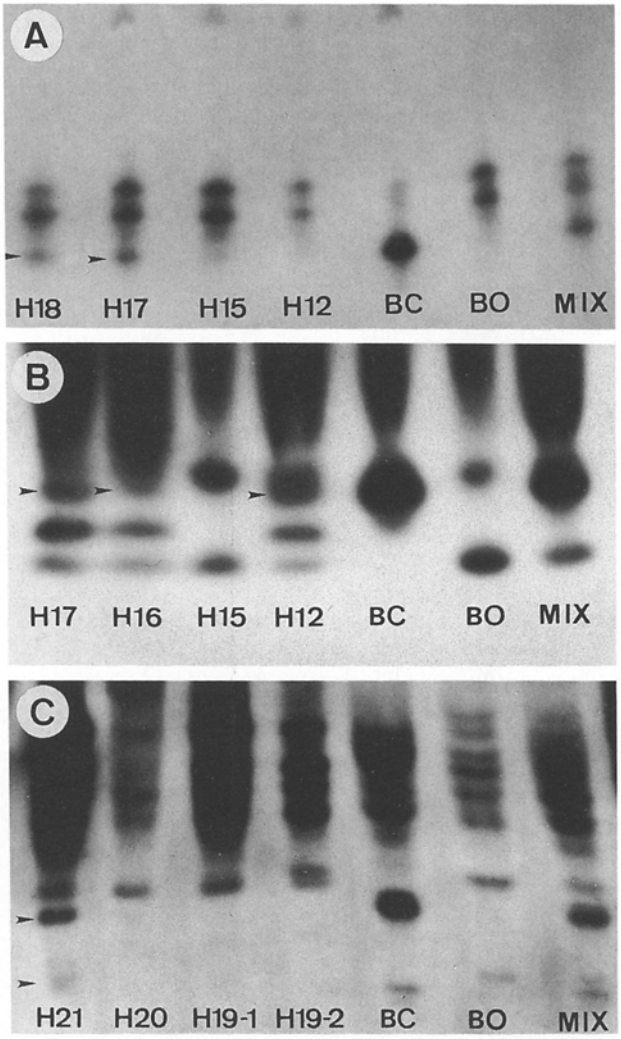


**Fig. 2 A–C.** Isozyme profiles of the hybrids and the parents. *B. oleracea* var. capitata cv. Nakawase rubi (BO), *B. campestris* (BC) var. rapa cv. 77b (**A, B**), var. pekinensis cv. CR-strong (**C**), and the mixture of *B. campestris* and *B. oleracea* (MIX) extracts; **A** Leucin aminopeptidase; **B** Acid phosphatase; **C** Esterase, *Arrow*; *B. campestris*-specific band

In the hybrids classified as the Int. type, the characteristic of *B. campestris* such as hairy comb on the leaf was noted, whereas those hybrids resembling *B. oleracea* did not exhibit these traits. However, each hybrid could be distinguished from *B. oleracea* by minor differences in morphology and by their dark green leaves (Fig. 1)

*2. Isozyme analysis*

Figure 2 shows the profiles of three isozymes examined. Some of the hybrids lost *B. campestris*-specific bands in all three of the isozymes examined, while *B. oleracea*-specific bands were retained in each hybrid except one (Table 1).

In LAP, two slow migrating bands of *B. oleracea* and one fast migrating band of *B. campestris* were detected in most of the hybrids. However, the *B. campestris*-specific band was not found in some of the hybrids (Fig. 2 A).

Banding patterns of APS and EST were more complex, but bands specific to either parent could be identified. In APS, a pattern identical to *B. oleracea* was found in some hybrids. In EST, a strong *B. campestris*-specific band was not present in some of the hybrids.

In summary, 10 of 22 hybrids exhibited all the parental bands in the three isozymes studied, 10 lost *B. campestris* bands in one or two isozymes and one lost *B. campestris*-specific bands in all of the three isozymes. One exceptional hybrid, H2, lost both *B. oleracea* and *B. campestris* bands in APS, suggesting the occurrence of alteration in APS expression. These results suggested that some of the *B. campestris* chromosomes were eliminated in most of the hybrids studied, due to X-ray irradiation prior to cell fusion.

*3. Chromosome analysis*

The presence of the small satellites of *B. oleracea* and the large satellites of *B. campestris* confirmed the hybrid nature of the plants obtained in this study (Fig. 3). In addition, morphologically altered chromosomes such as fragmented or reconstructed chromosomes were often observed in the hybrids (Fig 3 A). They were not found in either parent or in the symmetric hybrids studied previously (Terada et al. 1987 b).

The chromosome number of the hybrids was variable ranging from 30–74, and 20 of 22 hybrids studied were aneuploids. This is in contrast to the symmetric hybrids in which the majority of the hybrids contained 56 chromosomes (2 × *B. oleracea* + *B. campestris*) and only one aneuploid was found among the ten hybrids studied (Terada et al. 1987 b). From these and the results of isozyme analysis and the morphology, we assumed that most of the aneuploid hybrids resulted from elimination of *B. campestris* chromosomes from the hybrids initially possessing two *B. oleracea* genomes ($2 \times 18 = 36$) and one *B. campestris* genome (20).

Based on the assumption that elimination of *B. campestris* chromosomes occurred in the hybrids originally having 56 chromosomes, the number of the eliminated *B. campestris* chromosomes was calculated (Table 1). At 10 kR, 1–4 chromosomes were eliminated, whereas at 20 and 30 kR, 10–15 chromosomes appeared to be lost in the hybrids. In addition, hybrids containing the complete genome of *B. campestris* were detected at 10 and 30 kR. However, at higher X-ray doses, 60 and 80 kR, further increase in the number of eliminated chromosomes was not found although symmetric hybrids did not appear in these groups.

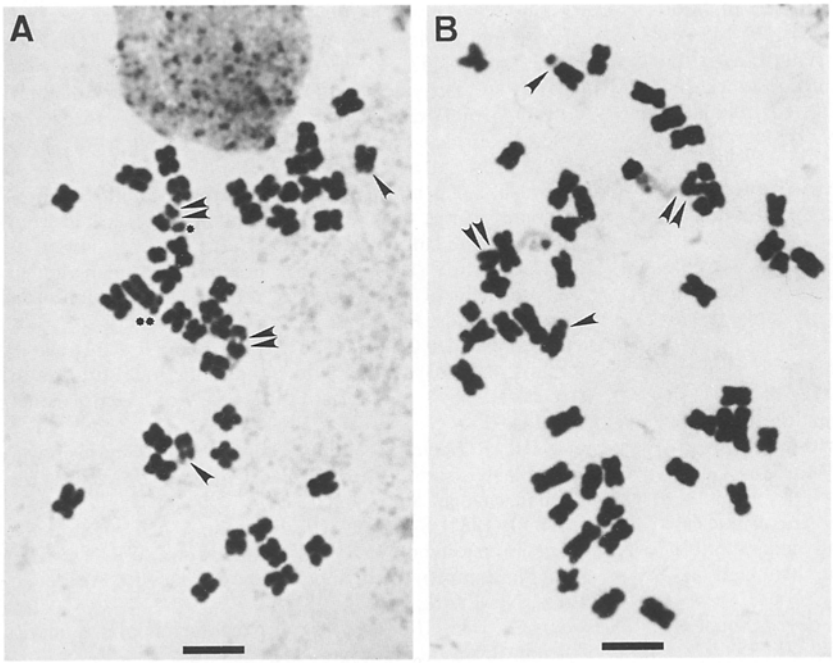


**Fig. 3 A and B.** Chromosome analysis of the hybrids. **A** A hybrid (H8) of *B. oleracea* var. capitata cv. Shikidori and *B. campestris* var. pekinensis cv. CR-strong, having 45 chromosomes. **B** A hybrid (H20) of *B. oleracea* var. capitata cv. Nakawase rubi and *B. campestris* var. pekinensis cv. CR-strong having 48 chromosomes. *Arrows* indicate satellites (*B. oleracea, single arrowhead*; *B. campestris, double arrowheads*). *Asterisks* indicate abnormal chromosomes (fragmented type, *single asterisk*; reconstructed type, *double astersks*). Bar = 5 $\mu$m

## Discussion

The morphology together with the results of the isozyme and chromosome analyses suggested that asymmetric somatic hybrids of *B. oleracea* and *B. campestris* could be obtained by X-irradiating *B. campestris* protoplasts prior to cell fusion. Reasons for the observation that the majority of the hybrids contained two *B. oleracea* genomes and 8–15 *B. campestris* chromosomes are not clear. One possibility is that specific combinations of *B. oleracea* chromosomes and *B. campestris* chromosomes were favored for shoot regeneration. The low frequency of shoot regeneration from the hybrid calluses might point to this possibility. Furthermore, the fact that increase of X-ray dose from 20–80 kR did not stimulate further elimination of chromosomes may be due to selection of hybrid calluses whose chromosome constitutions were suited for shoot regeneration. These should be clarified by analyzing the chromosomes of the hybrids at the callus level rather than at the plant level.

Among the three characters analyzed, there was a correlation between the morphology and the isozyme banding pattern (Table 1). Those hybrids having the *B. oleracea*-type morphology tended to lack the *B. campestris* isozyme bands (H9, 10, 15, 16, 19, 20). Between the chromosome number and the morphology or between the chromosome number and the isozyme pattern, no correlation was observed. For example, the H5 lost only two *B. campestris* chromosomes, but showed *B. oleracea*-type morphology. With regard to the isozyme pattern, some lost the *B. campestris*-specific bands for one to three isozymes (H6, 8, 9, 10, 12, 13, 15, 16, 19, 20, 21) and the others lost none of them (H6, 11, 14, 17, 18, 22). Nevertheless, they contained a similar number of chromosomes. These results may suggest random elimination of the chromosomes in each hybrid. More detailed examination of *B. campestris* chromosomes in the hybrids should be performed to further clarify the nature of the chromosome elimination in these asymmetric somatic hybrids. An analysis of the chromosomes of the hybrids by in situ hybridization using *B. campestris*-specific DNA probes is in progress.

The preliminary examination of the fertility of the hybrids showed that it was generally low when they were selfed. Although immature fruits were often produced, normal seed development was blocked in most of the plants. Nevertheless, when the hybrids were crossed by tetraploid *B. oleracea*, progeny seeds were obtained from most of the hybrids. This supports our conclusion that the hybrids contain the two complete genomes of *B. oleracea* plus a part of the *B. campestris* genome. The seeds will be further analyzed in the future.

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