DEVELOPMENT OF INTERSPECIFIC HYBRIDS BETWEEN *Brassica oleracea AND B. campestris* ADAPTED TO THE TROPICS

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ABSTRACT

We have developed interspecific hybrids between *Brassica oleracea* L. and *B. campestris* L. through ovule culture, which have seed vernalization characteristics, by introducing the seed vernalization character of *B. campestris* into *B. oleracea*. Most of the traits in the interspecific hybrids were intermediate between those of the female and the male parents. Chromosome number of the F_1 hybrids after chromosome doubling by colchicine was 38. The most common configuration was 19 bivalents at metaphase in pollen mother cells which contained both of the parental genomes (AACC).

The hybridization was confirmed also in the F_2 hybrids through cytological examination and isozyme analysis. The F_2 amphidiploid hybrids could be vernalized through low temperature treatment at 5°C for 3 weeks at the germination stage. The number of days to flowering was slightly longer than that in the male parent of *B. campestris*. Proline content in the leaves of the interspecific hybrids was nearly similar to that of the parents, which are heat-tolerant. These hybrids may be heat-tolerant and suitable for cultivation in the tropics.

Introduction

Interspecific crosses in *Brassica* have much impact and are very useful tools for the development of new vegetables and oil seeds. Recently interspecific or intergeneric hybridization through embryo culture has been frequently used in breeding programs of the *Cruciferae* to incorporate disease resistance (Namai *et al.*, 1980) and other economically important traits from one species to another (Hossain *et al.*, 1988b). However, success in the interspecific hybridization is limited, and it is more difficult when cabbage is used as the female parent (Hossain *et al.*, 1987).

In the tropics, seed production of some *Brassica* species, particularly cabbage (*B. oleracea* L. var. *capitata*) is not possible due to the insufficiently low temperature, though this is an important and popular vegetable in those areas (Harajada and Wein, 1987). As for the vernalization response, cabbage belongs to the plant-vernalization type (Friend, 1985 ; Shinohara, 1959). It requires low temperature exposure after completion of the juvenile phase for flower initiation, namely 5°C for 3 to 6 weeks at the 7-9 leaf stage depending on the cultivars (Heide, 1970). Therefore, seed production of cabbage remains a major constraint in the tropics.

On the contrary, Chinese cabbage (*B. campestris* L. var. *pekinensis*) belongs to the seed-vernalization type and the sensitivity to low temperature starts immediately after seed germination. Besides, the temperature for the flower induction in Chinese cabbage is higher than that in cabbage (Elers and Wiebe, 1984). It seems useful for the tropics to breed seed-vernalized interspecific hybrids between cabbage and Chinese cabbage. These would be artificial *B. napus* species that may be vernalized at the germination stage. In

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Brassica, however, barriers to interspecific hybridization include embryo abortion or abnormal embryo growth due to the lack of endosperm development (Raghavan and Srivastava, 1982).

Investigations aimed at transferring physiological traits such as seed vernalization response from one species to another are rare, as the response seems to be controlled by polygenes (Kagawa, 1971). Therefore, previous attempts to accelerate flowering in *Brassica* had emphasized the application of hormones to plants, which was not effective (Suge and Takahashi, 1982).

A useful parameter to evaluate the tolerance to high temperature stress is the proline content in plants which may act as a selection criterion for stress tolerance (Amberger and Obendorfer, 1988).

The present report describes successful attempts to develop seed-vernalized interspecific hybrids, by introducing the seed vernalization trait of Chinese cabbage into cabbage. This method is considered to be useful for overcoming the seed production constraints in *Brassica* in the tropical and sub-tropical areas.

Meterials and methods

Plant materials

The materials used in the present experiment consisted of *Brassica oleracea* L. var. *capitata* cv. Yoshin (cabbage), *B. oleracea* var. *alboglabra* Bailey cv. Senyo shirobana (Chinese kale) and *B. campestris* L. var. *pekinensis* cv. Kenshin (Chinese cabbage). All are heat-tolerant cultivars.

Ovule culture

The parental lines were crossed in a glasshouse at 20±5°C under natural day light conditions in April, 1987 and September, 1988. Hybrid capsules were collected 22 days after pollination, surface-sterilized with 70% (v/v) ethanol for a few seconds and with a solution of sodium hypochlorite containing 0.5% (w/v) active chlorine for 10 min, and were then rinsed 3 times with sterile water. More than one hundred fertilized ovaries were used to collect ovules for each treatment. The ovaries were dissected, and the ovules collected were explanted onto a modified MS medium containing Murashige and Skoog's major elements (1962) and Ringe and Nitsch's minor elements and organic supplements (1968), and supplemented with coconut water (10% v/v), casein hydrolysate (300 mg/l), NAA(0.1 mg/l) and kinetin (0.1 mg/l). This medium had been developed by Hossain *et al.* (1988a). Developed hybrid plantlets at about 30 days after explantation were transplanted onto hormone-free modified MS medium for further growth, and finally into pots. The F_1 interspecific hybrids were amphihaploid and sterile. Therefore, a few drops of colchicine (0.3% w/v) solution were applied for 3 days each to the shoot tip of the hybrids at the young stage to make their chromosome double. The F_2 seeds were collected from the chromosome doubled hybrids after selfing to investigate the introduction of seed-vernalization characteristics.

Cytology of the interspecific hybrids

The chromosome number of the interspecific hybrids was counted in root tip cells. The root tips were pretreated with 2 mM 8-hydroxyquinoline for 4 h at 20°C before fixation in 3 : 1 absolute ethanol : glacial acetic acid for 24 h. After fixation, the root tips were washed and treated for 60 min at 37°C with an enzyme solution containing 4% (w/v) cellulase Onozuka RS, 1% (w/v) pectolyase Y-23, 7.5 mM KCl and 7.5 mM EDTA, and then stained with 2% (w/v) aceto-orcein for 20 min as described previously (Hossain *et al.*, 1988a). Chromosomes were observed in ten metaphase cells in hybrid plants.

The meiotic behavior of the chromosomes was examined in pollen mother cells (PMC) using 1% (w/v) aceto-carmine for staining, and the chromosome configuration

was determined at the metaphase in colchicine-treated hybrid plants. Pollen viability of the hybrids was examined using 1% (w/v) aceto-carmine for staining, and stainable pollen grains were regarded as viable. In each plant 1000 pollen grains were observed.

Isozyme analysis

One gram of fresh leaves from the interspecific hybrid plants and their parents were crushed thoroughly in 1 ml of cold extraction buffer in chilled mortars. The extraction buffer was prepared with 0.1 M Tris (pH 7.2), 1% β-mercaptoethanol, 1% Triton X-100 and 5% polyvinylpyrrolidone. The homogenate was covered with a section of Kimwipe, and the extract was absorbed into 10×5 mm paper wicks placed on the Kimwipe.

Polyacrylamide gels for electrophoresis were prepared in combination of 44.4% acrylamide and 1.2% N, N, methylene-bis (Bis) for the running gel with a gel buffer of 0.5 M Tris (pH 8.8), and in combination of 10% acrylamide and 2.5% Bis for the stacking gels with a gel buffer of 0.5 M Tris (pH 6.8). In both types of gels 60% glycine was added. The electrode buffers consisted of 50 mM Tris with 373 mM glycine. The gels were first run at 80 v, and the voltage was increased 1 h later to 160 v and kept constant until the tracking dye migrated to the lower end of the gels. The gels were then taken out and stained for phosphoglucoisomerase (PGI) activity according to Arús and Orton (1983).

Low temperature treatment

Selfed F_2 seeds of interspecific hybrids were collected from the cross-combinations of 'Yoshin' (cabbage)×'Kenshin' (Chinese cabbage) and 'Senyo shirobana' (Chinese kale)×'Kenshin' (Chinese cabbage) and surface-sterilized with a solution of sodium hypochlorite (10% v/v) for 15 min, and then rinsed 3 times with distilled water. The treated seeds were sown into a vermiculite medium. Immediately after germination, the sprouts were grown for 3 weeks at 5 C or 15 C under 16 h/day of illumination with a fluorescent light at 3000 lux in a growth chamber. The control seedlings were grown at 23±2 C under natural daylight conditions of spring in a glasshouse. Ten seedlings were used for each treatment. After completion of the low temperature treatment, all the treated plants were grown in a glasshouse as in the case of the control.

Proline determination

Leaf samples weighing 0.5 g were collected from the hybrids and their parents grown at 20 C and 30 C in a temperature-controlled glasshouse. The samples were homogenized in 5 ml of 3% sulfosalicylic acid, and the homogenates were centrifuged at 3000 rpm for 20 min. Two ml of the supernants was reacted with 2 ml of acid-ninhydrin and 2 ml of glacial acetic in a test tube for 1 h at 98 C, and the reaction was terminated in an ice bath. The reaction mixture was extracted with 8 ml of toluene, vigorously shaken for 15-20 sec. Two milliliter extract from the toluene phase was used for reading at the absorbance at 520 nm at room temperature, with toluene as a blank. Proline content was determined on a fresh weight basis from a standard curve, using L-proline according to the method described by Bates *et al.* (1973).

Results and discussion

Breeding of interspecific hybrids

In total 17 and 13 interspecific hybrid plants were obtained through *in vitro* ovule culture from the cross combination of 'Yoshin' (cabbage)×'Kenshin' (Chinese cabbage) and 'Senyo shirobana' (Chinese kale)×'Kenshin' (Chinese cabbage) respectively. The regeneration rate of the plantlets was 5.67% in 'Yoshin'×'Kenshin' and 4.33% in 'Senyo shirobana'×'Kenshin' from 300 explanted ovules.

Seed production ability of the amphidiploid F_1 hybrids is shown in Table 1. Pod setting after selfing was significantly lower than in the parents, though pollen fertility

Table 1 Seed production ability of the amphidiploid interspecific hybrids between *B. oleracea* and *B. campestris*

Hybrid/Parent	Selfed pod set (%)	Days to maturity	Length of pods (cm)	Number of seeds/ pod	Seed germination (%)
'Yoshin'×'Kenshin'	47±3.27[1]	59±1.63	6.1±0.25	9.8±0.24	90
'Senyo shirobana'×'Kenshin'	55±1.63	48±2.45	5.7±0.33	8.0±0.16	89
'Yoshin' (cabbage)	89±3.26	74±3.26	6.8±0.08	15.0±1.63	95
'Senyo shirobana' (Chinese kale)	84±1.63	78±4.08	5.4±0.33	18.0±0.82	90
'Kenshin' (Chinese cabbage)	85±2.45	35±1.63	3.4±0.16	7.3±0.24	92

1 standard error.

exceeded 80% in both cross-combinations. The other characters such as number of days to seed maturity, length of pods and number of seeds per pod in the hybrids were intermediate between those in the female and the male parents.

Morphology of the hybrid plants

Morphology of the F_1 interspecific hybrid of 'Senyo shirobana'×'Kenshin' and the parents is shown in Fig. 1. Most of the traits of the F_1 hybrid plant were intermediate between those of the female and the male parents (Fig. 1 A-C). The main distinguishable characteristics of the hybrids of 'Senyo shirobana'×'Kenshin' and 'Yoshin'×'Kenshin' were observed in the leaf. The shape and waxiness of the leaf were markedly different compared with the parents (Table 2).

The F_1 hybrids of 'Yoshin'×'Kenshin' showed yellow flowers, whereas the F_1 hybrids of 'Senyo shirobana'×'Kenshin' had white flowers (Table 2). The flower size of the hybrids was intermediate between that of the parents. Occasionally the hybrids of 'Senyo shirobana'×'Kenshin' exhibited chimera flowers or yellow color flowers after chromosome doubling by colchicine application. These phenomena were related to pollen fertility and seed set. The completely yellow flowers of the chromosome doubled hybrids of 'Senyo shirobana'×'Kenshin' exhibited a higher pollen fertility than the others.

Cytology and isozyme analysis

The chromosome number of the hybrids of 'Yoshin'×'Kenshin' and 'Senyo shirobana'×'Kenshin' was 19. Therefore, these hybrids seem to be amphihaploid containing both of the parental genomes (AC). After chromosome doubling by colchicine treatment, the amphidiploids had 38 chromosomes and formed 19 bivalent chromosomes in pollen mother cells (Fig. 2 A).

PGI isozyme banding patterns of the parents and the hybrids are shown in Fig. 2 B. The isozyme bands of the F_1 hybrids of 'Senyo shirobana'×'Kenshin' showed a combination of the bands of both parents and one additional band. The isozyme bands of the F_2 plants showed a similar banding pattern.

Transfer of seed-vernalization characteristics from *B. campestris* to *B. oleracea*

All the F_2 hybrid plants and their parents failed to bloom in the control during the experimental period of 150 days, except that 60% of the plants bloomed in cv. Senyo shirobana. The hybrids of 'Yoshin'×'Kenshin' which were treated for 3 weeks at 5°C bloomed 115 days after sowing, while their male parents bloomed 77 days after sowing. Their female parents, however, did not bloom even when subjected to the same treatment (Table 3 ; Fig. 3 A, B). Only 40% of the F_2 hybrids of 'Yoshin'×'Kenshin' bloomed through seed vernalization for 3 weeks at 15°C. These findings indicate that the treatment at 15°C was less effective for flower induction in these hybrids than that of 5°C. These results suggest that the seed vernalization character of 'Kenshin' was transferred into the

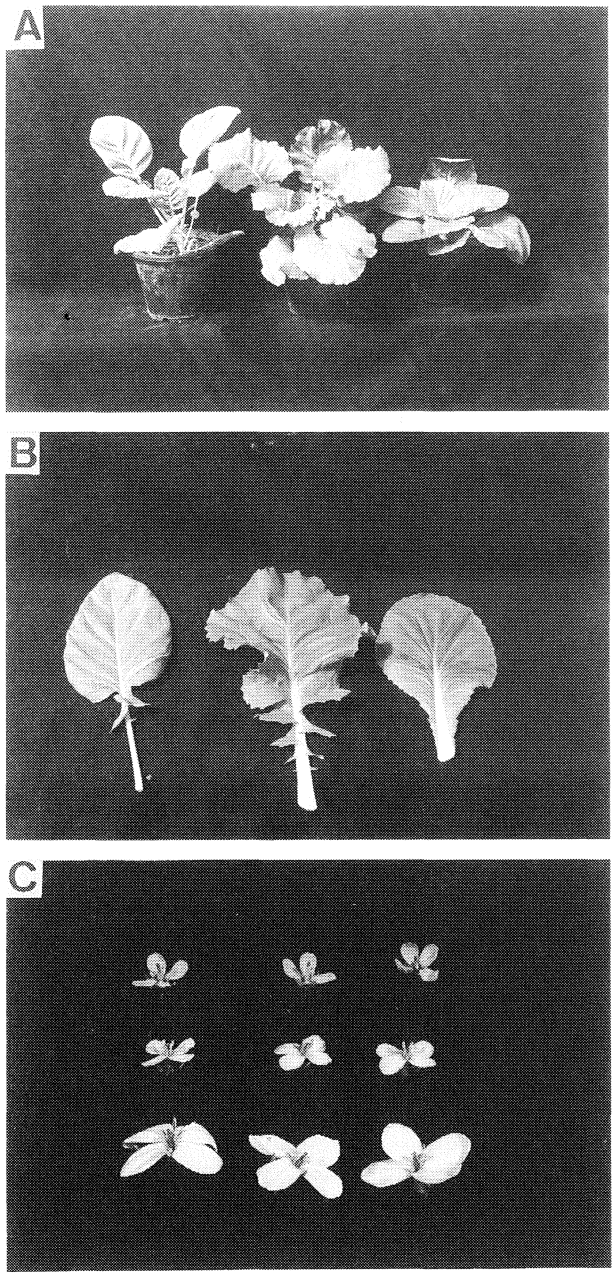

Fig. 1 Plant morphology of the interspecific F_1 hybrid of 'Senyo shirobana'×'Kenshin'
(A) the interspecific hybrid (center) shows a morphology intermediate between the female (left) and male (right) parents ; (B) the leaf of the hybrid (center) has distinct lobes, in comparison to those of the female (left) and male (right) parent ; (C) size of florets of the chromosome doubled F_1 hybrid (middle row) was intermediate between that of the female (lower row) and male (upper row) parent.

Table 2 Plant morphology of the interspecific hybrids and their parents in *Brassica*

Hybrid/Parent	Leaf character		Flower character	
	Leaf-edge	Waxiness	Color	Size
'Yoshin'×'Kenshin'	lobed	moderate	pale yellow	medium
'Senyo shirobana'×'Kenshin'	lobed	moderate	white	medium
'Yoshin' (cabbage)	entire	waxy	yellow	large
'Senyo shirobana' (Chinese kale)	entire	waxy	white	large
'Kenshin' (Chinese cabbage)	entire	not waxy	yellow	small

Table 3 Response to seed vernalization of the interspecific hybrids and their parents in *Brassica*

Temperature/Character	'Yoshin'×'Kenshin'	'Senyo shirobana'×'Kenshin'	'Yoshin'	'Senyo shirobana'[3]	'Kenshin'
5℃					
Days to bolting	104.00±3.27[1]	80.00±4.90	—[2]	101.00±2.45	71.00±7.35
Days to blooming	115.00±2.45	89.00±4.08	—	107.00±2.45	77.00±4.90
Plant height at anthesis (cm)	41.35±4.08	36.40±2.45	—	50.50±1.22	29.80±1.63
Leaf number at anthesis	34.30±2.87	15.35±1.37	—	9.50±0.50	26.00±2.16
Flowering (%)	100	100	—	100	100
15℃					
Days to bolting	134.00±2.45	103.00±5.72	—	90.00±1.63	105.00±5.72
Days to flowering	141.00±3.27	110.00±4.08	—	100.00±1.63	112.00±4.90
Plant height at anthesis (cm)	23.80±1.63	45.40±6.09	—	44.65±7.59	27.67±7.58
Leaf number at anthesis	41.00±4.08	17.30±0.83	—	9.00±0.58	25.35±3.09
Flowering (%)	40	100	—	100	100

1 Standard error.
2 No flowering after being subjected to vernalization treatment at germination stage.
3 Sixty percent of the plants bloomed in the control during the experimental period and the average of days to flowering was 107 days after sowing.

hybrid of 'Yoshin'×'Kenshin'.

The hybrids of 'Senyo shirobana'×'Kenshin' which were treated for 3 weeks at 5℃, bloomed 89 days after sowing. The blooming time was intermediate between that of the parents subjected to the low temperature treatment. It remains to be determined whether 'Senyo shirobana' (*B. oleracea* var. *alboglabra*) responded to seed vernalization, as 60% plants bloomed even in the control during the experimental period and the average number of days to flowering was 107 after sowing. It is considered that this plant was susceptible to growing temperature after the low temperature treatment, as this plant blooms readily in winter in the tropics. In the F_2 hybrids of 'Senyo shirobana'×'Kenshin', the blooming rate reached 100% even through the treatment at 15℃, presumably because the female parent did not require too low a temperature for flowering.

Free proline content in leaves

When the plants were grown at 20℃, the proline content in the leaf of the hybrids between 'Yoshin' and 'Kenshin' was 3.88 μ moles/g fresh weight (FW). This content was intermediate between that of the parents (Fig. 4). It increased to 5.02 μ moles/g FW under high temperature stress conditions at 30℃. Similar results were found in the hybrids between 'Senyo shirobana' and 'Kenshin'. The content was 3.47 μ moles/g FW under non-stress conditions at 20℃, and it increased to 4.50 μ moles/g FW under high temperature stress conditions at 30℃. Venekamp and Koot (1988) reported that the increase of the proline content in the vegetative parts was 10-to 25-fold in bean (*Vicia faba* L.) under water stress. In the present experiment, the increase of the proline content

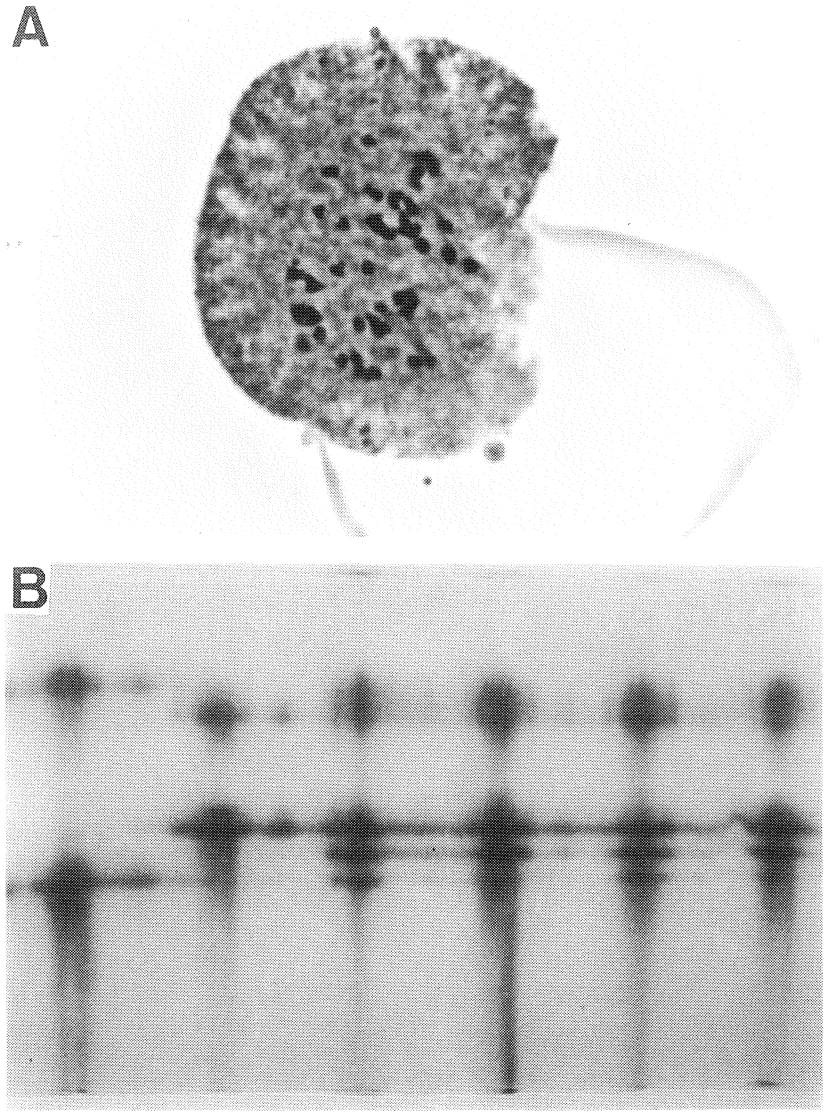

Fig. 2 Cytology and isozyme analysis of the interspecific hybrids

(A) 38 chromosomes in PMC at metaphase in the chromosome doubled F_1 hybrid of 'Yoshin'×'Kenshin' (1000×) ; (B) PGI isozyme banding pattern of the amphihaploid hybrid F_1 and the F_2 hybrid of 'Senyo shirobana'×'Kenshin' and their parents in polyacrylamide gel, 'Senyo shirobana' track 1 (from left to right), 'Kenshin' track 2, F_1 track 3 and F_2 tracks 4 to 6. The F_1 and F_2 hybrids showed all the bands of both their parents and a heterodimeric band peculiar to the hybrids. Anode is towards the top of the figure.

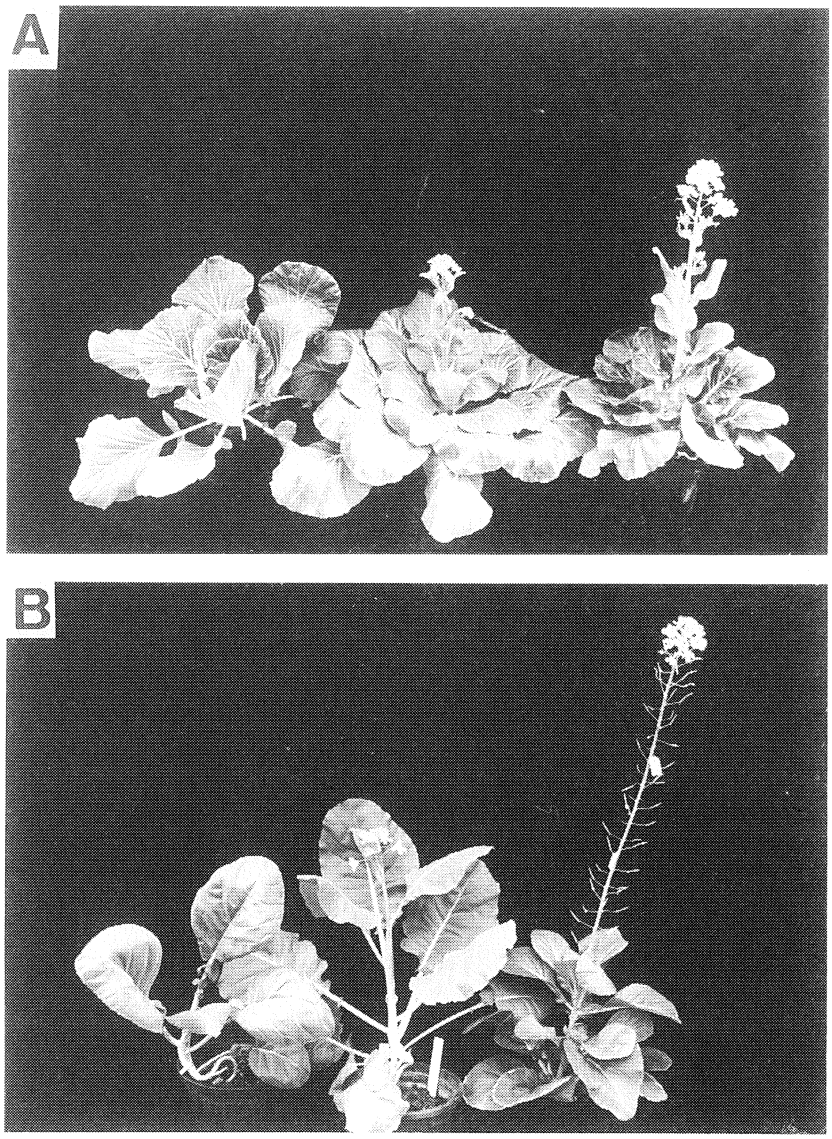

Fig. 3 **Seed vernalization response of the F_2 interspecific hybrids between *B. oleracea* and *B. campestris* and their parents**
The plants were exposed to low temperature at 5°C for 3 weeks at the germination stage : (A) the interspecific hybrid of 'Yoshin'× 'Kenshin' (center) was induced to flower in the same way as the male parent (right), while the female parent (left) formed a leaf-head ; (B) the interspecific hybrid of 'Senyo shirobana'× 'Kenshin' (center) bloomed about 10 days later than the male parent (right), while the female parent (left) did not bloom.

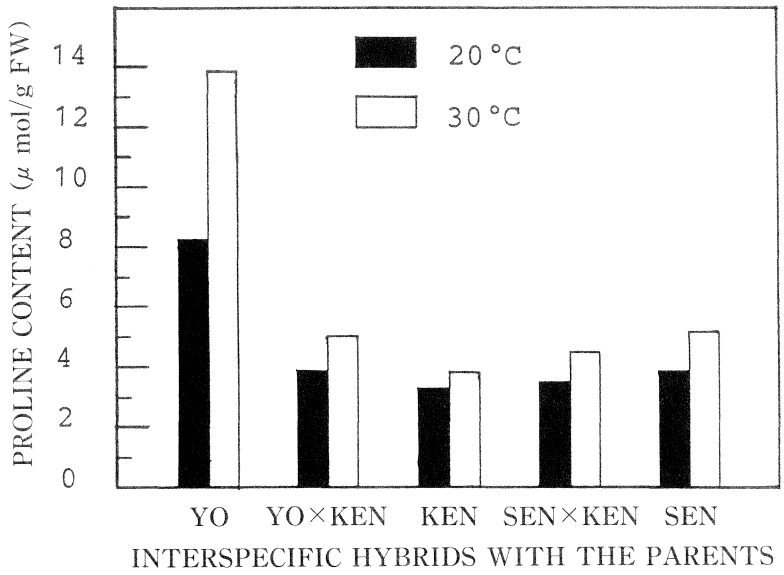

Fig. 4 Free proline content in leaves of the interspecific hybrids and their parents grown at 20°C and at 30°C
From left to right 'Yoshin' (YO), 'Yoshin' (YO)×'Kenshin' (KEN), 'Kenshin' (KEN), 'Senyo shirobana' (SEN)×'Kenshin' (KEN) and 'Senyo shirobana' (SEN).

in the hybrids of both groups, however, was less than 2-fold under high temperature stress. This result indicates that the interspecific hybrids of 'Yoshin'×'Kenshin' and 'Senyo shirobana'×'Kenshin' were heat-tolerant, as their proline content was not significantly increased even under high temperature stress.

Conclusion

Successful production of seed-vernalized interspecific hybrids has been achieved through *in vitro* ovule culture, after crossing growing plant-vernalized *B. oleracea* with seed-vernalized *B. campestris*. This technique is considered to be useful for the development of seed-vernalized hybrids which may contribute to overcome the seed production barriers in some *Brassica* species, particularly cabbage in the tropics.

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Discussion

Thongjiem, M. (Thailand) : Normally it is not difficult to obtain seeds of Chinese cabbage through open pollination in many countries. What is the main purpose of your studies ? Do you have any other plan to use the results obtained ? Is the quality of your hybrid comparable to that of pure Chinese cabbage, for instance it is known that the edible parts of Chinese kale are not as tender as those of Chinese cabbage.

Answer : I also mentioned in my report that the temperature for flowering induction in Chinese cabbage is higher than that of cabbage. Our objective was to develop seed-vernalized interspecific hybrids by transferring the seed vernalization trait of Chinese cabbage into cabbage. We have developed a newly synthesized cultivar of the heading type for our breeding programs which will be very close to cabbage morphologically with seed vernalization characteristics. The quality of the hybrids is under investigation. We do hope that they will exhibit the juiciness and softness of Chinese cabbage along with the keeping quality and agronomical traits of Chinese

kale.

Yui, S. (Japan) : Could you find any green plant vernalization type plants in the F_2 progeny population ? Why did you use Chinese kale as there is no problem for seed production in the tropical areas.

Answer : We could not find any green plant vernalization plants in the F_2 progeny in our cross-combinations. However, there were some variations, for instance days to flowering. We used Chinese kale as a breeding material to introduce the heat tolerance gene and early flowering trait to develop a newly synthesized cultivar that would be suitable for the tropics and that could be cultivated during the hot and humid summer in Japan.