Varietal Differences in Embryogenic and Regenerative Ability in Microspore Culture of Chinese Cabbage (*Brassica rapa* L. ssp. *pekinensis*)

Yasuhisa Kuginuki¹⁾, Koji Nakamura²⁾, Ken-ichi Hida¹⁾ and Hiroaki Yosikawa¹⁾

¹⁾ National Research Institute of Vegetables, Ornamental Plants and Tea, Ano, Mie 514-23, Japan

²⁾ Asahi Seed Co., Ltd., Kamisobue, Bisai, Aichi 494, Japan

Summary

Varietal differences in embryogenesis and plant regenerative ability from isolated microspores of Chinese cabbage, *Brassica rapa* L. ssp. *pekinensis*, were investigated. There were significant differences (< 1 %) among the varieties in total embryoid yield and regenerative embryoid yield per 1×10^5 microspores. Tropical varieties had high embryogenesis and regenerative ability. 'Hsifu Early 30 Days' showed the highest embryoid yield and 'Homei' showed a higher efficiency of plant regeneration from microspores. However, domestic Japanese varieties showed very low embryogenesis and plant regeneration ability in microspore culture. Though 'Hsifu Early 30 Days' showed a higher embryoid yield than 'Homei', the latter showed more efficient plant regeneration from embryoids than the former. There was no correlation between the total embryoid yield and the percentage of embryoids with plant regeneration. These results suggest that plant regeneration from embryoids is controlled by some genetic factors different from those controlling embryogenesis.

In order to introduce high regenerative ability to domestic Japanese varieties, microspores isolated from the F₁ hybrid of 'Homei' × 'Nozaki No. 2' (domestic Japanese variety) were cultured. Doubled haploid lines, which had both high regenerative ability and characteristics comparatively similar to domestic Japanese varieties, could be selected among these regenerated plants. If high regenerative lines with domestic Japanese characteristics are developed, it is expected that the application of microspore culture to practical plant breeding will become easier for the breeding of Chinese cabbage in Japan.

Key Words: *Brassica rapa*, embryogenesis, haploid, microspore culture, plant regeneration, varietal differences.

Introduction

The haploid method of breeding is useful and effective for genetic fixation because the doubled haploid is theoretically homozygous. Since the successful anther culture of *Brassica* species was first described by Keller *et al.* (1975) and Thomas and Wenzel (1975) for *B. napus* L., this technique has been applied to several other *Brassica* species (George and Rao 1982, Ockendon

1985, Govil *et al.* 1986, Arora and Bhojwani 1988, Sato *et al.* 1989c).

Microspore cultures have several advantages over anther cultures, including the absence of regeneration from somatic tissue and a significant reduction in labor requirer (Swanson *et al.* 1987). Since haploid plants were first obtained from isolated microspores of *B. napus* by Lichter (1982), the technique of microspore culture has been studied intensively in order to improve embryoid yield and plant regeneration frequency (Chuong *et al.* 1988, Fan *et al.* 1988, Sato *et al.* 1989b, Takahata and Keller 1991). The information gained from such studies is valuable for rapeseed breeding programs considering the use of haploidy (Siebel and Pauls 1989).

For the application of microspore culture to the practical breeding of *Brassica* species, often unpredictable and very low embryoid yield has been a serious problem. The genotype is thought to be the most important factor affecting embryogenesis of *Brassica* species (Chuong *et al.* 1988, Takahata and Keller 1991). For Chinese cabbage (*B. rapa* L. ssp. *pekinensis*), which is one of the most important vegetables in Japan, there have been few reports of successful microspore culture (Sato *et al.* 1989a, Wakui *et al.* 1994). Though there have been some successes in the microspore culture of *B. rapa* L. (syn. *B. campestris* L.) (Baillie *et al.* 1992, Cao *et al.* 1994, Ferrie *et al.* 1995), varietal differences in the frequency of plant regeneration from isolated microspores have not been reported. Therefore, it is difficult to apply microspore culture to the practical breeding of Chinese cabbage. In the present study, varietal differences in the embryogenesis ability and plant regeneration from isolated microspores of Chinese cabbage were investigated.

Materials and Methods

First experiment

Twenty-five varieties of Chinese cabbage were examined (Table 1), including the semi-heading type, cylindrical (Michihili) type, ovate type (Li 1981) and tropical type (Saito 1947). The tropical type, which was not reviewed by Li (1981), is cultivated mainly in southern China and Southeast Asia. It has a small round head, and small, thick, glossy and dark green leaves with comparatively narrow midribs. The bolting of tropical varieties is earlier than that of other types. All of the ovate varieties in this experiment were domestic Japanese varieties which had been developed in Japan.

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Table 1. Varietal differences in embryoid yield and regenerative embryoid yield in microspore culture¹⁾

Variety	Type ²⁾	Origin ³⁾	Geometric mean of number of embryos		Geometric mean of the number of embryos with regenerated plants		Percentage of embryos with plant regeneration
Homei	TR	AVRDC	25.7	ab ⁴⁾	13.8	a ⁴⁾	54.0
Changpuh Early	TR	AVRDC	6.7	cdef	6.1	ab	91.0
Kensui	TR	SC	23.4	abc	5.7	abc	24.4
Hsifu Early 30 Days	TR	AVRDC	52.7	a	4.7	bcd	8.9
Santo Sai	SH	NIAR	8.2	bcde	4.7	bcd	57.3
Formosa 45 Days	TR	AVRDC	1.8	efgh	1.8	cde	100.0
Homei Yungching	TR	AVRDC	15.9	abc	1.0	de	6.3
Tokyo Takenoko	CY	SC	11.3	bcd	1.0	de	8.8
Linzinqing	CY	China	4.5	cdefg	1.0	de	22.2
Hsiasheng 35 Days	TR	AVRDC	1.8	fgh	0.4	de	22.2
Muso	OV	SC	0.7	gh	0.4	de	57.1
Pikaichi	TR	SC	4.0	defgh	0.0	e	0.0
Nozaki No.2	OV	SC	2.2	defgh	0.0	e	0.0
Chihili 70	CY	SC	0.0	h	0.0	e	0.0
Qingmaye	CY	China	0.0	h	0.0	e	0.0
Chosen	OV	NIAR	0.0	h	0.0	e	0.0
Hatsukaze	OV	SC	0.0	h	0.0	e	0.0
Kaga	OV	NIAR	0.0	h	0.0	e	0.0
Matsushima New No.2	OV	NIVOT	0.0	h	0.0	e	0.0
New Riso	OV	SC	0.0	h	0.0	e	0.0
Osho	OV	SC	0.0	h	0.0	e	0.0
Simoyama Chitose	OV	NIAR	0.0	h	0.0	e	0.0
Kashin	SH	NIAR	0.0	h	0.0	e	0.0
Kenryu	TR	SC	0.0	h	0.0	e	0.0
Tainung 40 Days	TR	AVRDC	0.0	h	0.0	e	0.0

¹⁾ Two plants for each variety were examined. Before taking the logarithm of the number of embryos per 1×10^5 microspores, the number of embryos was increased by 1 in each flower bud class and then statistical analyses were performed. Therefore these geometric means were 1 less than the calculated ones.

²⁾ CY : cylindrical, OV : ovate, SH : semi-heading, and TR : tropical type.

³⁾ Introduction from China, Asian Vegetable Research (AVRDC) in Taiwan, National Institute of Agrobiological Resources (NIAR) in Japan, National Research Institute of Vegetables, Ornamental Plants and Tea (NIVOT) and seed companies (SC) in Japan.

⁴⁾ Values in the same column following the same letter are not significantly different at the 1% level, according to LSD.

These varieties have a tight and ovate head, and comparatively rough, thick and green leaves with wide white midribs.

Seeds of the donor plants were sown in a greenhouse in early February, where the minimum temperature was kept above 10 °C. After 2 weeks, they were transplanted into a glasshouse without heating, and flowering occurred during March and April. Two plants for each variety were used in the microspore culture.

The microspore culture was carried out according to the method of Sato *et al.* (1989b) with modifications. Young flower buds were sampled from donor plants and classified, according to bud-length into four groups : 2.0 to 2.3, 2.4 to 2.7, 2.8 to 3.1 and 3.2 to 3.5 mm. Isolated microspores from each bud-length group were cultured separately, because the microspore developmental stage influences embryogenesis (Fan *et al.* 1988). The buds were sterilized in sodium hypochlorite solution contain-

ing 2 % active chlorine for 15 min. They were washed with sterilized-distilled water 4 times and gently pestled in BM-10 medium (Table 2), using a mortar 8 cm in diameter and a pestle 13 cm long. BM-10 medium, which was a modified B medium (Sato *et al.* 1989b), was adjusted to pH 6.0 and filter-sterilized. The suspension was filtered through a 63 µm stainless steel mesh and collected in a centrifuge tube. BM-10 medium was added and then the mixture was centrifuged at $120 \times g$ for 3 min. The centrifuging and washing steps were repeated 3 times, then the microspores were resuspended in BM-10 medium at a 1×10^5 microspores/ml. Two milliliters of this suspension was plated into each of three 60×15 mm sterilized plastic petri dishes. These were incubated at 33 °C in darkness for 24 hr and then maintained at 22 °C in darkness.

After 3 weeks of culture, the number of embryos at each developmental stage was counted. The embryos

Table 2. Composition of the initiation medium (BM-10¹⁾) for microspore culture in *Brassica rapa* L.

Compound	mg/l	Compound	mg/l
KNO ₃	62.5	myo-Inositol	100
KH ₂ PO ₄	62.5	Nicotinic acid	5
Ca (NO ₃) ₂ · 4H ₂ O	250	Glycine	2
MgSO ₄ · 7H ₂ O	62.5	Pyridoxine HCl	0.5
FeSO ₄ · 7H ₂ O	13.9	Thiamin HCl	0.5
Na ₂ EDTA	18.65	Biotin	0.05
H ₃ BO ₄	6.2	Folic acid	0.5
MnSO ₄ · 4H ₂ O	22.3		
ZnSO ₄ · 7H ₂ O	8.6	Glutathione	30
KI	0.83	L-Glutamine	800
Na ₂ MoO ₄ · 2H ₂ O	0.25	L-Serine	100
CuSO ₄ · 5H ₂ O	0.025		
CoCl ₂ · 6H ₂ O	0.025	Sucrose	100,000

¹⁾ BM-10 : The concentration of FeSO₄ · 7H₂O, Na₂EDTA and sucrose are modified and the plant growth regulators are omitted from Sato's medium (1989b). The pH of the medium was adjusted to 6.0 with KOH and filter-sterilized.

which grew into the torpedo or cotyledonary stage were transferred onto half-strength MS medium containing 1 % sucrose and 0.5 % Gellun Gum (Wako Pure Chemical Industries Ltd. Osaka) and incubated at 22 °C with a 16 hr light/8 hr dark photoperiod of cool white illumination at 50 μ mol/m²s. After 3 weeks, they were transferred onto half-strength MS medium with 1 % sucrose and 0.8 % agar. Regenerated plants were grown in vermiculite for about 2 weeks before transfer to soil. After 10 weeks of initiation culture, the number of embryoids with regenerated shoots, which grew more than 2 cm, was recorded.

The mean numbers of total embryoids and embryoids with regenerated shoots were calculated for each donor plant as follows. Among four groups classified by flower bud-length, two groups showing more regenerative embryoid yield than the other two were selected, and then the geometric mean was calculated. Before taking the logarithm of the number of embryoids, the number of embryoids was increased by 1 in each group. Two plants for each variety were examined and each donor plant was examined twice. Then the geometric mean was calculated for each variety. After these mean values had been transformed to logarithms, statistical analysis was performed.

Second experiment

Two breeding lines 'A9302' and 'A9303' with domestic Japanese characters and high regenerative ability were produced. Isolated microspores from the hybrid of 'Homei' × 'Nozaki No. 2' (domestic Japanese variety) were cultured and 50 regenerated plants were obtained. Ten out of these 50 plants were selected for high frequency of plant regeneration, and 8 of the 10 were selected for seed production by bud pollination. Two lines, 'A9302' and 'A9303', were selected among these 8 selfed lines

Table 3. Varietal differences in embryoid yield and regenerative embryoid yield in microspore culture¹⁾

Variety	Type ²⁾	Origin ³⁾	Geometric mean of number of embryoids	Geometric mean of the number of embryoids with regenerated plants	Percentage of embryoids with plant regeneration
A9303 ⁴⁾	OV	NIVOT	101.1 a ⁵⁾	44.7 a ⁵⁾	44.2
A9302 ⁴⁾	OV	NIVOT	106.1 a	44.2 a	41.7
Homei	TR	AVRDC	43.2 a	22.2 a	51.4
CCPL No.4 ⁶⁾	TR	NIVOT	3.2 b	1.4 b	43.8
Orange Queen	OV	SC	1.4 bc	1.4 b	100.0
Muso	OV	SC	1.0 bc	1.0 bc	100.0
Utage 70	OV	SC	2.7 b	0.7 bc	25.9
Ryutoku	OV	SC	1.4 bc	0.4 bc	28.6
CCPL No.1 ⁶⁾	OV	NIVOT	0.0 c	0.0 c	0.0
Daifuku 75	OV	SC	0.0 c	0.0 c	0.0
Horai No.3	OV	SC	0.0 c	0.0 c	0.0
Kanko	OV	SC	0.0 c	0.0 c	0.0
Kogetsu 70	OV	SC	0.0 c	0.0 c	0.0
Kukai 65	OV	SC	0.0 c	0.0 c	0.0
Satokaze	OV	SC	0.0 c	0.0 c	0.0
W-1116	OV	SC	0.0 c	0.0 c	0.0

¹⁾ Two plants for each variety were examined. Before taking the logarithm of the number of embryoids per 1×10^5 microspores, the number of embryoids was increased by 1 in each flower bud class and then statistical analyses were performed. Therefore these geometric means were 1 less than the calculated ones.

²⁾ OV : ovate and TR : tropical type.

³⁾ Introduction from Asian Vegetable Research (AVRDC) in Taiwan, National Research Institute of Vegetables, Ornamental Plants and Tea (NIVOT) and seed companies (SC) in Japan.

⁴⁾ Breeding line from a cross of 'Homei' and 'Nozaki No.2' selected for embryogenic ability.

⁵⁾ Values in the same column following the same letter are not significantly different at the 1% level, according to LSD.

⁶⁾ Chinese cabbage parental line (CCPL).

for their close similarity to the Japanese varieties. To compare with these two lines, eleven Japanese F₁ hybrid varieties, 'Homei' (tropical type) introduced from the Asian Vegetable Research and Development Center (AVRDC), and two Chinese cabbage parental lines (CCPL), 'CCPL No. 1' and 'CCPL No. 4', which are resistant to clubroot disease, were used (Table 3).

Seeds of donor plants were sown in a growth chamber under a 16 hr photoperiod of 200 μ mol/m²s with mercury-vapor and sunbeam lamps and a day/night temperature of 20 °C/15 °C. After 2 weeks they were vernalized in a cold room at 4 °C and under a 16 hr photoperiod of 20 μ mol/m²s for 3 weeks. These plants were then transferred to the growth chamber as described above. Microspore culture was carried out as in the first experiment.

Results

Some varieties showed varying embryoid yields and regenerative embryoid yields among the groups classified by flower bud-length in the same donor, and the bud size giving the best embryoid yield differed among the

genotypes (data not shown).

First experiment

'Hsifu Early 30 Days' and 'Changpuh Early' showed differing efficiency of embryogenesis between donors. The better donor plant had about five times as many embryoids as the poorer one for 'Hsifu Early 30 Days' and about three times as many for 'Changpuh Early'. Other accessions showed relatively stable efficiency between donors (data not shown).

There were significant differences ($< 1\%$) in the total embryoid yield and the regenerative embryoid yield among the varieties (Table 1). Thirteen out of 25 varieties formed embryoids in microspore culture. Embryogenesis from microspores was similar among these 13 varieties. After 2 to 3 days of culture, the microspores swelled and then in some the first nuclear divisions were observed. Globular embryoids were formed after 7 to 10 days of culture. The embryoids reached the torpedo and cotyledonary stages after 2 to 3 weeks.

'Hsifu Early 30 Days' showed the highest embryoid yield, followed by 'Homei' introduced from AVRDC and 'Kensui' (Table 1). 'Kensui' had the same characteristics as the varieties introduced from AVRDC. In 8 out of 10 tropical varieties, embryogenesis was frequently observed, and some of them showed a high embryoid yield. Among other types, 'Tokyo Takenoko' (cylindrical type) showed the highest embryoid yield, followed by 'Santo Sai' (semi-heading type). In comparison, the domestic Japanese varieties showed very low embryoid yield.

In 'Hsifu Early 30 Days', which showed the highest embryoid yield in this experiment, 151 embryoids per 1×10^5 microspores were formed in the 2.8 to 3.1 mm bud-length group (data not shown). However, the frequency of plant regeneration from microspores was low (Table 1). On the other hand, 'Homei', 'Changpuh Early' and 'Kensui' showed a higher frequency of plant regeneration from microspores.

The correlation coefficient between the number of total embryoids and the percentage of embryoids showing plant regeneration was $r = -0.24$.

Second experiment

1. Breeding process of 'A9302' and 'A9303'

Isolated microspores from the hybrid of 'Homei' $\times$ 'Nozaki No. 2' (domestic Japanese variety) were cultured and this hybrid had a higher embryogenesis and regenerative ability than those of 'Homei' (Kuginuki *et al.* 1991) and 50 regenerated plants were obtained. These 50 plants exhibited differences in embryoid yield ranging from 116 embryoids per 1×10^5 microspores to 0 and regenerative embryoid yield ranging from 51 embryoids to 0. Nine plants showed no embryoid in these 50 regenerated plants. Ten plants which showed more regenerative embryoids than 'Homei' were selected out of the 50 plants. Out of these 10 plants, 8 plants which had an

ability of seed production similar to 'Nozaki No. 2' by bud pollination were selected (data not shown). Two lines 'A9302' and 'A9303', which had wider midrib and later bolting than the other 8 selfed lines, were selected.

2. Embryogenesis and regeneration ability

'Homei' showed varying efficiency of embryogenesis among donors (data not shown). The better donor plant had about three times as many embryoids as the poorer one. Other accessions showed relatively stable efficiency among donors.

There were significant differences ($< 1\%$) in embryoid yield and regenerative embryoid yield among the varieties (Table 3). Eight varieties out of 16 formed embryoids. 'A9302' and 'A9303' showed the largest number of total embryoids and embryoids with regenerated shoots (Table 3). In this experiment, the highest embryoid yield was 227 per 1×10^5 microspores of 'A9302' in the 2.4 to 2.7 mm bud-length group and the yield of 'A9303' in the 2.4 to 2.7 mm group was 201. These numbers were higher than those of 'Homei', which had the higher embryogenic ability between the parents. Compared with these breeding lines, the Japanese F_1 hybrid varieties had low frequencies of both embryogenesis and plant regeneration.

The correlation coefficient between the number of total embryoids and the percentage of embryoids showing plant regeneration was $r = -0.27$.

Although plant growth conditions differed between Experiments 1 and 2, some varieties of the tropical type were found to have higher embryogenic ability and Japanese varieties showed the lower embryogenesis efficiency in both experiments (Tables 1 and 3).

Discussion

The embryoid yield from isolated microspores differed markedly among Chinese cabbage varieties (Tables 1 and 3). Such an influence of donor plant genotype on embryogenesis has been reported in *B. napus* (Chuong *et al.* 1988) and *B. oleracea* (Takahata and Keller 1991). Sato *et al.* (1989c) reported that the 'kenshin type' (a tropical type) had an extremely high embryogenic ability, and Japanese varieties of 'Matsushima New No. 2' and 'Nozaki No. 2' had the lowest embryogenesis efficiency among Chinese cabbage varieties in anther culture. Our results for microspore culture agreed with their results for anther culture.

The highest embryoid yield reported so far for *Brassica* vegetables has been 530 per 1×10^5 microspores of broccoli (Takahata and Keller 1991), 86 for cabbage (Kuginuki *et al.* 1993) and 250 for Chinese cabbage (Sato *et al.* 1989b). In the present experiment, the highest embryoid yield was 227 in 'A9302' and 201 in 'A9303' (data not shown). These breeding lines were therefore considered to have the high embryogenic ability among *Brassica* vegetables.

This is the first study of varietal differences in regenerative ability from microspore-derived embryos of *Brassica* vegetables. The frequency of plant regeneration also differed significantly among the varieties of Chinese cabbage in microspore culture (Tables 1 and 3). These results showed that the efficiency of plant regeneration is influenced by genotype. Although 'Hsifu Early 30 Days' showed the highest embryoid yield in the first experiment, the efficiency of plant regeneration from embryoids was low (Table 1). In comparison, 'Homei' showed a higher efficiency of plant regeneration. The low correlation coefficient between the number of total embryoids and the percentage of embryoids showing plant regeneration indicated that plant regeneration from embryoids was controlled by genetic factors different from those controlling embryogenesis. Although the frequency of plant regeneration from embryoids has been improved in some *Brassica* species, it is still low (Keller *et al.* 1987, Takahata and Keller 1991). Therefore, tissue masses abnormally developed from primary embryoids often need to be subcultured many times in order to induce normal shoots from microspore-derived embryoids. These drawbacks may override the general effectiveness of the haploid system in terms of time and labor in breeding programs (Chuong *et al.* 1988). On the other hand, 'A9302' and 'A9303' induced few abnormal embryoids and showed a high efficiency of plant regeneration without subculturing many times. Such high regenerative ability was considered to be useful in the application of microspore culture to practical plant breeding.

The domestic Japanese varieties exhibited low abilities of both embryogenesis and plant regeneration in microspore culture. This limited the application of microspore culture to practical breeding. Therefore, we tried to develop breeding lines which have agronomic characters similar to domestic Japanese varieties and high regenerative ability similar to tropical ones. Some reports have indicated that the F_1 has a high ability of androgenic embryogenesis in *B. napus* (Siebel and Pauls 1989) and *B. rapa* (Kuginuki *et al.* 1991). The results for the hybrid 'Homei' × 'Nozaki No. 2' agree with previous studies. Hence it is easy to obtain many regenerated plants from the microspores of the hybrids. Ten out of the 50 regenerated plants showed a higher number of embryoids with plant regeneration than 'Homei', which has the higher embryogenic ability between the parents. 'A9302' and 'A9303' were selected out of these 10 plants for their comparatively desirable characteristics and high seed production, and showed higher regenerative ability from microspores than 'Homei' (Table 3). From this result, it is suggested that 'Homei' had the variation of embryogenic and regenerative ability derived from microspores and the higher ability was fixed in 'A9302' and 'A9303'.

We are continuing to develop breeding lines which have similar characteristics to domestic Japanese varieties without losing their high regenerative ability.

Judging from the correlation coefficient between the number of regenerative embryoids and the number of days from sowing to bolting ($r = -0.23$), or the width of midrib in the largest leaf ($r = -0.27$) in the 50 regenerated plants, there is probably no marked genetic linkage of characteristics of domestic Japanese varieties and high regenerative ability. Therefore, it is thought that high regenerative ability can be introduced into varieties with domestic Japanese characteristics. As these lines are developed, it is expected that the application of microspore culture will become easier for the practical breeding of Japanese varieties of Chinese cabbage.

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