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Determination of Microspore Population to Obtain High Frequency Embryogenesis in Broccoli (*Brassica oleracea* L.)

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To develop an efficient microspore culture system for broccoli, the effect of the developmental stage of the cultured microspore population was clarified, and embryogenic microspores were selected by percoll density gradients. The mean number of microspore nuclei was more effective than bud size for the determination of the developmental stage and microspore population that would be embryogenic. A microspore population having an average of 1.54-1.84 nuclei produced a high yield of embryos. Microspores selected by percoll (28/36%) produced about 3-fold the number of embryos of those that were not selected.

Introduction

Isolated microspore culture of *Brassica* is a valuable tool for genetic manipulation as well as for haploid breeding[1-4]. The development of an efficient microspore culture system is prerequisite to these objectives. Although the optimal conditions for *B. napus* microspore embryogenesis have been revealed[5-9], those of other *Brassica* species have not been sufficiently clarified. Takahata and Keller reported efficient embryogenesis on *B. oleracea* microspore culture[10]. They revealed some conditions such as culture condition, genotype and bud size. However, the yield of embryos was lower when compared with that of *B. napus*[8,9].

In order to further improve the microspore culture system of *B. oleracea*, several factors influencing embryogenesis must be optimized. Of these, it is important to determine the developmental stage of the microspore population which is to undergo embryogenesis. Cytological studies in *B. napus* indicated that microspores from late uninuclate to early binuclate have embryogenic ability[11,12]. And fractionation using percoll density gradients was reported to be effective for the selection of microspores having a high frequency of embryogenesis in rapeseed[13]. However, in *B. oleracea* microspore culture, little is known about criteria or easy methods for identifing and selecting a microspore population capable of inducing efficient embryogenesis.

In this study, we adopted the mean number of microspore nuclei for estimating the developmental stage of the broccoli microspore population, and investigated the effect of developmental stage on microspore embryogensis. In addition, selection of embryogenic microspores by fractionation using a percoll density gradient was carried out.

Materials and Methods

1. *Plant materials*

One strain of broccoli (*B. oleracea* L. var. *italica* B 31-18) was used. The plants were propagated

on B 5[14] agar-solidified medium by aseptic cutting in a growth cabinet at 25°C with 16 h/day photoperiod (30 mE m^{-2} s^{-1}). After 3-4 weeks of subculture, the plants were transferred to soil and grown in an uncontrolled-environment green house. Then they were transferred to a growth chamber at 13/8°C (day/night) with natural light conditions at the commencement of bolting.

2. *Microspore culture*

Flower buds 3.7-5.0 mm in length were collected. The protocol of microspore culture was executed as previously described[10]. After three weeks of culture, the embryo yield was examined using a stereo microscope.

3. *Observation of microspore developmental stage*

At the time that microspores were plated in a petri dish a small sample was taken from each bud, stained with 2 μg/m*l* of DAPI (4′, 6-diamidino-2-phenylindole), and observed using ultraviolet irradiation with a fluorescent microscope[11]. To estimate the developmental stage of the microspore populations, we used the mean number of microspore nuclei.

4. *Selection of microspores by percoll density gradients*

Microspores were isolated from flower buds 4.0-5.0 mm in length. Following the second washing, 0.5-1.0 m*l* of the microspore suspension was overlaid on 28 and 36% percoll solution with 13% sucrose[13], and centrifuged at 200 g for 6 min. The microspores at each interface of the gradient were separately collected. After the microspores were washed once with 1/2 NLN-13 medium[10], they were cultured as described before.

Results and Discussion

1. *Effect of microspore developmental stage on microspore embryogenesis*

Bud size is generally used as a good criterion for judging the optimal develomental stage of microspores for embryogenesis. However, as shown in **Fig. 1**, the identical size bud did not always reflect the same developmental stage. In our previous report, microspores isolated from buds 4.0-5.0 mm in length had the best response[10]. In this study, the microspore population from this size buds of one individual consisted of uninuclate to trinuclate stage, but that of another individual was

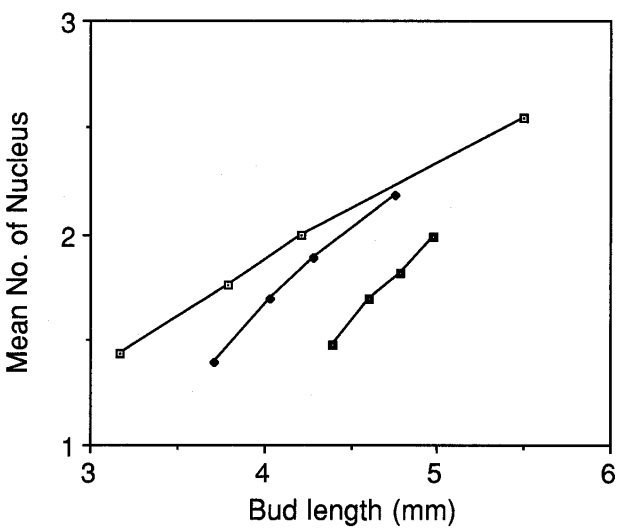

Fig. 1 Relationship between mean number of nuclei of microspore population and bud length. Three lines were derived from different individuals and ages.

Table 1. Effect of microspore developmental stage on microspore culture of broccoli.

Mean no. of microspore nucle*[1]	Bud size (mm)	No. of embryos per petri dish*[2]	Frequency*[3] (%)
1.00	3.8	65.3± 2.8	0.07
1.15	4.0	91.3± 4.2	0.09
1.41[§]	3.7	11.2± 2.7	0.01
1.54	4.4	710.0±36.2	0.71
1.68	4.6	986.6±77.7	0.99
1.73[§]	4.0	511.4±20.4	0.51
1.84	4.8	791.5±79.6	0.79
1.98[§]	4.2	0.4± 0.2	0.00
2.00	5.0	44.5± 2.1	0.05

*[1]More than 200 microspores were examined.
*[2]1×10^5 microspores were cultured in a 60×15 mm petri dish.
*[3]% of cultured microspores developing into embryos.
[§]Buds were collected at different time.

composed of uninuclate and binuclate stages (**Fig. 1**). This result supports the suggestion that optimum bud size including embryogenic microspores varied with the growing conditions and age of the plants[15], and indicates that more clear criterion is needed to develop an efficient microspore culture system.

The mean number of nuclei of the microspore population from 3.7-5.0 mm buds varied from 1.0 to 2.0 (**Table 1**). Embryogenesis depended on the developmental stage. A high yield of embryos was obtained with microspore populations having a mean nuclear number of 1.54-1.84. The population with a mean nuclear number of 1.68 showed the best response (0.99% of cultured microspores induced embryogenesis). The microspore populations whose developmental stages were earlier and later had a lower rate of embryogenesis. On the other hand, optimal bud size including embryogenic microspores was different when collected at different times. For example, the microspore population from 4.0 mm buds collected at one time had a mean nuclear number of 1.15 nuclei and showed poor embryogenesis, but microspores from the same size buds collected at other time had a mean nuclear number of 1.73 and showed high rate of embryogenesis. These results indicate that mean nuclear number is an accurate index for selecting embryogenic microspores.

Although in rapeseed, the optimal developmental stage for embryogenic response was reported to be the late uninuclate to early binuclate stage [11,12], it is difficult to differentiate this stage from the preceding stage. In addition, microspores from the identical bud were not synchronized. The index used in this study is a good criteria for the determination of microspore developmental stage and the selection of a microspore population in which embryogenesis can be induced. The present results indicated that a microspore population that is 50-80% binuclate had high embryogenic ability. This supports the suggestion that the optimal developmental stage is older than that usually handled for microspore culture, which consists of abundant late uninuclate and a few binuclate microspores[16].

2. *Selection of embryogenic microspore by percoll density gradients*

The frequency of embryogenesis varied among microspores collected from each interface. The microspores at interface between 28 and 36% percoll, which had a mean nuclear number of 1.58, produced significantly more embryos than those at other percoll levels (**Table 2**). This value was about three times that in which microspores were not selected. Microscopic observation showed

Table 2. Effect of percoll fractionation on microspore culture of broccoli.

Percoll (%)	Mean no. of microspore nuclei	No. of embryos per petri dish*1	Frequency*2 (%)
0/28	—	138.0± 22.9	0.138[b]
28/36	1.58	633.0±102.3	0.634[a]
36/	1.07	2.0± 0.7	0.002[b]
No selection	—	184.9± 37.9	0.185[b]

*1 1×10^5 microspores were cultured in a 60×15 mm petri dish.

*2 % of cultured microspores developing into embryos. Values followed by the same letter in a column are not significantly different, according to Duncan's multiple renge test ($p<0.05$).

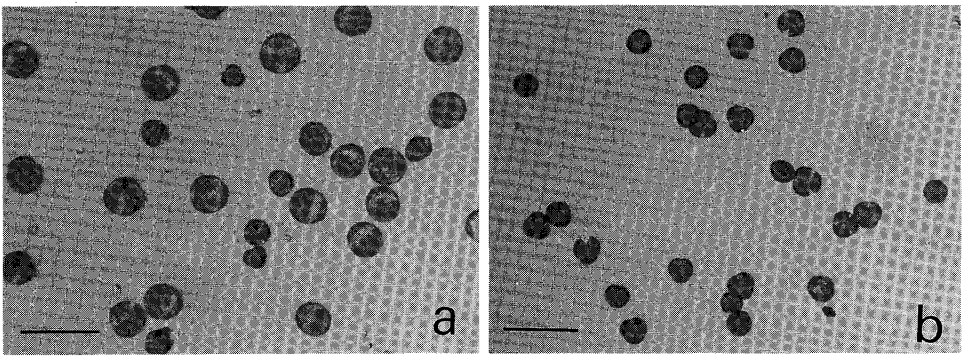

Fig. 2 Microspores obtained by percoll fractions of 28/36 (a) and 36/(b). Bar=40 μm.

that a large number of these microspores were characterized by their large and spherical form (**Fig. 2-a**). On the other hand, most of microspores that precipitated to the bottom of the 36% percoll, which exhibited small and trilobed form (**Fig. 2-b**), did not have embryogenic ability. This result indicates that fractionation using percoll density gradients is effective for the selection of embryogenic microspores. This agrees with the results obtained from *Nicotiana*[17,18] and rapeseed[13].

Selecting highly embryogenic microspores is a great advantage for genetic manipulation and developmental research. The present results suggest that selection using percoll density gradient on microspore population collected on the basis of mean number of nucleus will be more effective. Our recent experiments showed that microspores selected by percoll from microspore population having a 1.5-1.7 mean nuclear number produced the highest frequency of embryogenesis (1.4% of cultured microspores formed embryos).

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《和文要約》

ブロッコリー小胞子培養における効率的胚発生を得るための小胞子集団の選抜

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効率的なブロッコリー小胞子培養系を確立するため，小胞子の発達段階の影響とパーコール密度勾配遠心による小胞子の選抜について調査した．小胞子集団の発達段階を判断する基準および胚発生に最適な小胞子集団を選ぶための指標として、平均小胞子核数が蕾の長さより適していた．1.54-1.84 の平均小胞子核数を持つ小胞子集団が効率的な胚形成を示し，1.64 の時 0.99% と最も高い胚形成率を示した．また，パーコールで小胞子を選抜した結果，28/36% の境界の小胞子は平均核数 1.58 で選抜しないものより 3 倍以上の胚形成率を示した．