Effect of Low Temperature Pretreatment of Buds or Inflorescence on Isolated Microspore Culture in *Brassica rapa* (syn. *B. campestris*)

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The effect of low temperature pretreatment of buds or inflorescence on microspore culture for the production of haploids of *Brassica rapa* (syn. *B. campestris*) was examined. Incubation of the buds or the inflorescence at 4°C for 3 or 10 days before the culture of microspores induced efficient microspore embryogenesis. Pretreatment of flower buds was more effective than that of the inflorescence. Prolonged pretreatment up to 20 days promoted embryo induction. Microspores in the buds were examined for developmental stage before and after the pretreatment. Buds with a petal length to anther length ratio of about 0.5-0.7, which had microspores at the late unicellular stage, were collected and were used. All microspores were at the late unicellular stage before the pretreatment. The percentage of the microspores at the stage of late unicellular decreased during the pretreatment while the percentage of bicalar stage microspores with two unequal size nuclei increased. At the same time, a small but notable number of bicalar stage microspores with equal size nuclei, which is the first step of microspore embryogenesis, was observed after the pretreatment.

Key Words: *Brassica rapa*, haploid, microspore culture, embryogenesis, low temperature pretreatment.

Introduction

A number of factors, such as genotype, growing conditions of donor plant, microspore developmental stage, medium constituents, and culture conditions, affect microspore embryogenesis. High temperature (30-35°C) treatment for 1-3 days of culture before transferring to 25°C markedly stimulates embryogenesis in anther culture and microspores culture of *Brassica* species, (Keller and Armstrong 1979, 1983, Constantine *et al.* 1996, Takahata 1997), and is now routinely used in microspore and anther culture of Brassicas including *B. rapa* (syn. *B. campestris*) (Sato *et al.* 1989, Kuginuki *et al.* 1997, Zhang and Takahata 1999).

Pretreatment of the plants, inflorescence, or buds before culture of microspores or anthers might affect microspore embryogenesis. Pretreatments reported to be effective for *Brassica* species include decreased atmospheric pressure (Klimaszewska and Keller 1983), gamma irradiation (MacDonald *et al.* 1988), colchicine treatment (Zaki and Dickinson 1991) and ethanol stress (Pechan and Keller 1989). However, the results of low temperature pretreatment, although routinely used in microspore and anther culture of cereals (Constantine *et al.* 1996), are contradictory for *Brassica* species (Keller 1984).

In this study, we investigated the effect of low temperature pretreatment on the microspore culture of *B. rapa* and found that the pretreatment improved the ability of microspore embryogenesis. Microspores pretreated with a low temperature for a prolonged period (20 days) showed the same extent of embryogenesis as microspores pretreated for 3 or 10 days. We also observed microspore development during the low temperature pretreatment.

Materials and Methods

Plant materials

*Brassica rapa* (syn. *B. campestris*) “Tsubame” (Tohoku Seed Co., Ltd., Tochigi, Japan) was used throughout the experiments in this study. To investigate the genotypic difference, “W1116” (Watanabe Seed Co., Miyagi, Japan), “Harusakari” (Watanabe Seed Co.), “CR-Kanko” (Nippon Norin Seed Co., Tokyo, Japan), and “Shunraku” (Nippon Norin Seed Co.) were used. Seeds were sown in potted soil in a greenhouse. Seedlings about a week after germination were vernalized for 4 weeks at 4°C under continuous light (white fluorescent lamp, light intensity of 20 µmol m^{-2} S^{-1}). The vernalized plants were transplanted to plastic pots (27 cm diameter, 30 cm height) and were grown in a greenhouse at day and night temperatures of 23°C and 17°C respectively. The plants bolted and flowered after about one month.

Microspore culture

Flower buds 1.5-2.0 mm in width, with a petal length to anther length ratio (P/A) of about 0.5-0.7, were collected. While this stage of flower buds mainly corresponded to the late unicellular stage (1CL) of microspore, a small number...
of early bicellular microspores with nuclei unequal in size (2CU) were also present in each collected bud depending on growth conditions of the donor plants. Our preliminary experiments showed that the microspores of these flower buds (0.5-0.7 of P/A) had a high potential for embryogenesis.

The microspore culture procedure was based on Sato et al. (1989) with modifications. All media were filter sterilized using 0.22 μm filter units. Flower buds were surface sterilized in 0.1% benzalkonium chloride solution for 30 sec and then in calcium hypochlorite (1% w/v) solution for 15 min. The sterilized buds were rinsed three times with sterile distilled water. These buds were gently macerated in a petri dish (6 cm in diameter) containing 5 ml of modified B5 (Keller and Armstrong 1979) liquid medium with 13% sucrose and without L-glutamine, L-Serine or plant growth regulators at pH 6.0 (mB5-13) using a mortar. The suspen- 
sion of microspores was filtered through a 40 µm nylon mesh and the filtrate was centrifuged at 120 × g for 3 min. The precipitated microspores were washed three times with sterile distilled water and were stored for 0, 3, and 10 days at 4°C.

Two milliliters of the suspension were cultured in a sterilized plastic petri dish (6 cm diameter, 1.5 cm height), were incubated for 24 hr at 33°C, and then for 4 weeks at 25°C in the dark. Then the number of embryos was counted.

Low temperature pretreatment

The inflorescence was collected from each plant and cut to 10 cm in length. It was put into a beaker containing distilled water and were stored for 0, 3, and 10 days at 4°C in the dark. After the low temperature treatment, buds (P/A ratio of 0.5-0.7) were collected from the inflorescence, and microspores were isolated from the buds to conduct microspore culture. For the treatment of flower buds, buds including microspores at the late unicellular stage (P/A ratio of 0.5-0.7) were removed from the plants and were surface sterilized as described above. These buds were put into a petri dish containing 5 ml of mB5-13 medium, and then were stored for 0, 3, 7, 10, 15, and 20 days at 4°C in the dark. After this treatment, the microspores were isolated from the buds without surface sterilization and were cultured. In the experiments on the effect of the low temperature pretreatment, each treatment used five petri dishes containing microspores isolated from 25 flower buds each.

Cytological study

To investigate the effect of low temperature (4°C) on the development of microspores, the microspore stages of development were identified using 4,6-diamino-2-phenylindole (DAPI) stain and a fluorescent microscope (Fan et al. 1988, Hamaoka et al. 1991). Our preliminary experiments showed that the developmental stages of microspores of all 6 anthers from a single flower bud were highly synchro-

ized. Anthers were collected at 0, 7, 9, 14, 22 and 29 days after the start of the low temperature pretreatment of buds. The anthers were fixed in an ethanol:acetic acid (3 : 1, v/v) solution prior to staining with DAPI, and were transferred to a buffer (pH 4.4) containing 56 mM citric acid and 88 mM sodium phosphate. These anthers were placed on a glass slide, were squashed in a drop of DAPI solution (0.25 mg/l in the same buffer), and were observed under a fluorescent microscope. For each anther, more than 100 microspores were observed to identify developmental stages of microspores.

Results

Flower buds or inflorescence of B. rapa “Tsubame” before isolation of microspores were pretreated for 3 or 10 days at 4°C. The effectiveness of embryogenesis from the microspores was significantly improved with low temperature pretreatment (Table 1) except for the 3-day pretreatment of the inflorescence. The 10-day pretreatment of resulted in higher numbers of embryos with treatments of flower buds and inflorescence, but the treatment of buds was more effective. The low temperature pretreatment was also effective in all other varieties tested: “W1116”, “Harusakari”, “CR-Kanko”, and “Shunraku” (data not shown). The effects of the duration of the low temperature pretreatment on buds were examined. The number of embryos increased after pretreatment for 7-20 days in this study (Table 2). There was a difference in the number of embryos

<table>
<thead>
<tr>
<th>Duration of 4°C treatment (days)</th>
<th>Treated organ</th>
<th>No. of embryos/petri dish ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No treatment</td>
<td>2.0 ± 0.6 a</td>
</tr>
<tr>
<td>3</td>
<td>Inflorcescence</td>
<td>3.2 ± 1.1 a</td>
</tr>
<tr>
<td>3</td>
<td>Buds</td>
<td>8.2 ± 1.2 b</td>
</tr>
<tr>
<td>10</td>
<td>Inflorcescence</td>
<td>6.0 ± 1.2 b</td>
</tr>
<tr>
<td>10</td>
<td>Buds</td>
<td>31.5 ± 4.5 c</td>
</tr>
</tbody>
</table>

Table 1. Effect of low temperature (4°C) pretreatment of buds or inflorescence on embryo formation in microspore culture of B. rapa (syn. B. campestris) “Tsubame”

<table>
<thead>
<tr>
<th>Duration of 4°C treatment (days)</th>
<th>No. of embryo/petri dish ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.8 ± 1.4 a</td>
</tr>
<tr>
<td>3</td>
<td>14.5 ± 2.2 a</td>
</tr>
<tr>
<td>7</td>
<td>30.3 ± 4.8 b</td>
</tr>
<tr>
<td>10</td>
<td>20.4 ± 0.9 ab</td>
</tr>
<tr>
<td>15</td>
<td>37.5 ± 5.1 c</td>
</tr>
<tr>
<td>20</td>
<td>32.5 ± 3.4 c</td>
</tr>
</tbody>
</table>

Table 2. Effect of duration of low temperature (4°C) pretreatment by flower buds on embryo formation in microspore culture of B. rapa (syn. B. campestris) “Tsubame”

1) Values followed by the different letters in the same column are significantly different at 5% level by t-test.
Induced without pretreatment from experiment to experiment; such as 2.0 shown in Table 1 and 15.0 in Table 2. However, in each experiment, the effect of low temperature treatment was analyzed by the t-test.

The influence of low temperature (4°C) treatment on microspore development was observed. Table 3 shows the percentages of microspores at each developmental stage before and after various periods of low temperature treatment of flower buds. At the start of the treatment, all microspores in the buds were at the late unicellular stage. The percentage of the late unicellular stage microspores (1CL) decreased during the low temperature pretreatment, while the percentage of the bicellular stage microspores with two unequal size nuclei (2CU) increased. Further, a small but notable number of bicellular stage microspores with two equal size nuclei (2CE) was observed after low temperature pretreatment for 7 days or more. Tricellular stage microspores with two generative cells and one vegetative cell which seemed to be normal pollen were observed after low temperature pretreatment for 29 days.

The ratio of the length of petal to that of anther (P/A) was calculated before and after the low temperature pretreatment of buds. The P/A of buds before the pretreatment was 0.5-0.7, but that after the pretreatment of 29 days was 0.9-1.0.

**Discussion**

Inconsistent results of the effect of low temperature pretreatments of buds or inflorescence before microspore or anther culture on microspore embryogenesis in *Brassica* have been reported. Low temperature pretreatment improves microspore embryogenesis in *B. napus* (Lichter 1982), *B. juncea* (George and Rao 1982), and *B. oleracea* (Osolnik et al. 1993). However, low temperature treatment of the buds inhibits microspore embryogenesis in *B. campestris* (Keller et al. 1982) and *B. napus* (Dunwell et al. 1985). Our results showed that in the microspore culture of *B. rapa* (syn. *B. campestris*), low temperature pretreatments of buds or inflorescence had distinctly improved the ability of microspore embryogenesis (Table 1). These contradictory results might have been caused by the difference in the conditions of the treatment. In the cases of negative effects reported, buds were put in a sealed bottle (Dunwell et al. 1985) or a sealed plastic bag (Keller et al. 1982) during the pretreatment. However, in the studies where promotive effects were obtained, buds were put on a piece of wet cotton (Osolnik et al. 1993) or in a liquid medium (Lichter 1982) during the pretreatment. In the present study, buds were floated in a liquid medium, under the wet condition, the microspores probably remained viable for a few weeks.

We used longer treatments than any reports cited, and found that 7-20 days of pretreatment was effective for microspore embryogenesis. In the normal development of microspore to pollen, the late unicellular stage microspores divide asymmetrically and change to the bicellular microspores with the two unequal nuclei that have a big vegetative cell and a small generative cell. However, at embryogenesis of microspores, the late unicellular stage microspores divide symmetrically and change to bicellular microspores with two equal nuclei (Hamaoka et al. 1991). Under low temperatures, the percentage of late unicellular stage microspores decreased and the percentage of bicellular stage microspores with two unequal nuclei (2CU) increased almost to the same extent as the decrease in 1CL percentage. However, at the same time a small but notable number of the bicellular stage microspores with two equal nuclei (2CE) appeared. Thus, during low temperature treatment, most microspores slowly develop to normal pollen. At the same time a small number of microspores develop to embryos. This may explain the promotive effect of low temperature pretreatment on microspore embryogenesis. The mechanism by which low temperature pretreatment induces and promotes the symmetrical division of microspores is still unknown. Microspore embryogenesis is effectively induced with 25 µM colchicine treatment of microspore culture of *B. napus*; the cytoskeletons of microspores are disrupted by depolymerization of microtubules by exposure to colchicine, resulting in symmetrical division instead of asymmetric first pollen mitosis (Zhao et al. 1996). Microtubules are destroyed by low temperature treatment in tobacco

### Table 3. Percentages of developmental stage of microspores before and after low temperature treatment of buds of *B. rapa* (syn. *B. campestris* “Tsubame”)

<table>
<thead>
<tr>
<th>Duration of 4°C treatment (days)</th>
<th>Developmental stage of microspores (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1CL&lt;sup&gt;1)&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td>7</td>
<td>86.9 ± 9.1</td>
</tr>
<tr>
<td>9</td>
<td>54.4 ± 22.0</td>
</tr>
<tr>
<td>14</td>
<td>59.1 ± 23.9</td>
</tr>
<tr>
<td>22</td>
<td>63.8 ± 9.0</td>
</tr>
<tr>
<td>29</td>
<td>29.6 ± 4.1</td>
</tr>
</tbody>
</table>

<sup>1)</sup> unicellular late stage microspore.
<sup>2)</sup> bicellular stage microspore with two unequal size nuclei.
<sup>3)</sup> bicellular stage microspore with two equal size nuclei.
<sup>4)</sup> tricellular stage microspore with two generative cells and one vegetative cell.

Each value represents mean ± SE of 10 anthers.
BY-2 cells (Hasezawa et al. 1997). During the low temperature pretreatment in our study, the microtubules in the microspores might have been destroyed, which in turn might have induced symmetrical division of some microspores.

Pretreatment with a low temperature was less effective for the inflorescence than for buds. We used the P/A ratio of a bud as an index of the developmental stage of microspores in the bud. Buds with a P/A ratio 0.5-0.7 were optimal for microspore culture because their microspores were mainly at the late unicellular stage. For pretreatment with a low temperature, we collected buds with a P/A ratio 0.5-0.7. After pretreatment, the P/A ratio of the buds increased to 0.7-1.0. For the pretreatment of inflorescence, we collected buds with a P/A ratio of 0.5-0.7 after the pretreatment and cultured the microspores from the buds. If the buds of the pretreated inflorescence grew at nearly the same rate as those pretreated directly, the P/A ratio 0.5-0.7 of buds after the pretreatment must have been lower than 0.5-0.7 before the pretreatment. This might be why pretreatment of the inflorescence was less effective than that of buds.

Prolonged low temperature pretreatment of buds showed a promotive effect in *B. rapa*. Thus buds or inflorescence for microspore culture can be stored for up to three weeks under low temperatures; thus, concentration of work can be avoided. Low temperature pretreatment is routinely used for doubled haploid production in our breeding programs.

**Acknowledgments**

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**Literature Cited**


