Selection of *Brassica napus* L. embryogenic microspores by flow sorting

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**ABSTRACT**

Flow cytometry can be used to select and sort microspore subpopulations of *Brassica napus* cv. Topas. Data obtained from embryogenic microspore populations were used to identify potentially embryogenic microspores from developmentally heterogeneous microspore populations based on differences in forward light scatter and green autofluorescence. Culture enrichment for embryogenic microspores is possible. Frequencies of 8 and 14% microspore embryogenesis were obtained when selected 16 h and 72 h after culture initiation. This represents 5- and 13-fold increase in microspore embryogenesis compared to non-sorted controls.

**INTRODUCTION**

Flow cytometry has a wide range of applications in studies of cell population dynamics and characteristics. Advances in plant regeneration from single cell suspensions enhanced the value of applying flow cytometry to sort plant protoplasts, select for heterokaryon products from protoplast fusion experiments and subsequently regenerate plants (Redenbaugh et al. 1982; Alexander et al. 1985; Afonso et al. 1985; Glimelius et al. 1986; Pauls and Chuong 1987). Flow cytometry could also be useful for separation of haploid and diploid embryogenic plant cell populations based on nuclear DNA content (Arndt-Jovin and Jovin 1977). Moreover, transformation products from cocultivation or direct DNA uptake experiments could be quickly identified and selected if fluorescence markers were built into the vectors. Factors governing the flow cytometric analysis and sorting of plant cells have been investigated in detail by Harkins and Galbraith (1987).

Recent advances in *Brassica napus* microspore culture (Keller et al. 1988; Pechan and Keller 1988; Fan et al. 1988) allow us to examine the usefulness of flow cytometry for microspore culture manipulation. As the first step, it is important to ascertain whether and how efficiently flow sorting can differentiate between microspore subpopulations and select for microspores capable of undergoing embryogenesis.

**MATERIALS AND METHODS**

1. **Microspore culture**

Plants of *Brassica napus* cv. Topas were grown at 10/5°C day/night temperature, 16-h photoperiod, 500 µE m⁻² s⁻¹ light intensity and 50% relative humidity. Sterilized 3-5 mm buds were placed in a modified B5 medium as described by Keller and Armstrong (1977) but free of growth regulators and supplemented with 13% sucrose, macerated with a glass rod, released microspores filtered through a 44 µm Nitex screen and washed 3x by centrifugation in the B5-13% sucrose medium at 100 g for 3 min. Microspores were subsequently suspended in a modified Lichter medium (Lichter 1982), without growth regulators or potato extract, plated onto 60 x 15 mm (Falcon 1007) petri dishes and incubated in darkness at 32.5°C. Prior to cell sorting, microspores were pelleted at 200 g for 3 minutes and resuspended in a modified Lichter medium to a final concentration of 0.5 to 1.0 x 10⁶ microspores per ml. Microspores were sorted at 16 h and 72 h after culture initiation.

2. **Flow cytometry**

a) **Method for analysis:**

Flow cytometry was performed utilizing an EPICS C (Coulter Electronics, Hialeah, FL) instrument with a Coherent (Palo Alto, CA) 5-watt Innova 90 argon-ion laser tuned to 488 nm at 500mW. Green fluorescence was analyzed using a 525 nm interference filter and a 550 nm SP dichroic mirror in optical block two. In block one, 488 nm dichroic and a 575 nm interference filters were installed. The two additional filters utilized were a 515 nm long-pass and a 525 nm interference filters in optical blocks one and two, respectively. The signals were processed with a log-linear amplifier. Data analysis was performed with Coulter EASY 88 Data Management System (Coulter Electronics, Hialeah, FL). QUADSTAT program (version 2.1) was used for dual parameter light scatter data analysis.

b) **Method for sorting:**

Highly embryogenic microspore cultures, originating from specifically selected buds (Pechan and Keller 1988), were used to accumulate flow cytometry data on embryogenic microspores for 4 days subsequent to culture initiation. This
data was entered into the computer memory for subsequent selection of potentially embryogenic microspores from developmentally heterogeneous microspore populations.

Sorting bitmaps were set on logarithmic two parameter histograms of the relative fluorescence intensity versus forward light scatter. A vibrating piezoelectric quartz crystal tuned at the frequency of 32 kHz broke the flow stream into separate droplets as the stream passed the tip of the flow chamber and was just beyond the point of laser interrogation. Sheath fluid pressure differential was 13-15 psi. The flow tip utilized had a 76 um inner diameter. The hydrodynamics of the system is designed in such a way that every third droplet should contain a microspore. A microspore that met the bitmap specifications received a charge that was either negative or positive. As the droplets passed between the two electrostatically charged plates of the sort deflecting assembly, they were deflected to the respective reversely charged plates (left or right). A 0.8% saline solution was used as the electrolyte medium. At initial concentrations of 1 x 10^6 microspores per ml, the flow unit sorted approximately 300-500 microspores per second. In order to reduce the possibility of atmospheric contamination during sorting two additional procedures were adapted. Prior to sorting the decontamination procedure of the fluidic system by Redenbaugh et al. (1982), was followed with some modification. After operating the fluidic system for a minimum of 5 minutes with 70% ethanol, 13% sucrose solution was flushed through for a minimum of 2 minutes to eliminate any osmotic stress. The left and right cell collectors were also modified. Ten mm diameter plastic test tubes were cut to 8 cm height with a false bottom. This adapter permitted the collecting glass tube to be within 2.5 cm of the sort deflecting assembly, thus reducing the exposure of the biological material to laboratory atmosphere. It also ensured that microspores fell directly into the modified Lichter medium rather than onto a test tube wall. Although the saline solution was not sterile, only one out of nine experiments was contaminated.

Subsequent to flow sorting, microspores were pelleted by centrifugation (200 g for 3 min), resuspended in 1.25 ml of a modified Lichter medium and plated onto 60 x 15 mm (Falcon 1007) petri dishes. Microspores were incubated in darkness at 32.5°C up to day 4 after culture initiation and subsequently transferred to 25°C. The number of embryos formed were counted 21 days after culture initiation. Where appropriate, samples of microspores and proembryos were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma Co.), a DNA specific stain, for cytological observations (Pechan and Keller 1988). Microspore diameters were measured with an eyepiece micrometer mounted on a Zeiss microscope.

RESULTS AND DISCUSSION

Flow cytometry offers an efficient and quick way to classify and sort a heterogeneous population of microspores into a number of subpopulations (Fig. 1). Embryogenic microspores can be identified and sorted: such microspores are capable of undergoing normal embryogenic development and subsequently form plants. Thus 7.7% of the microspores formed embryos when selected at 16 h after culture initiation; at 72 h after culture initiation, 13.7% formed embryos (Tab. 1). This represents frequencies 5-13x higher than the unsorted controls. Flow sorting can thus be used to enrich for embryogenic microspores. The frequencies of embryo enrichment are comparable to the percoll gradient method (Fan et al. 1988).

Microspore selection based on green autofluorescence and forward light scatter, expressed on a log scale, were the optimal criteria to differentiate microspores into subpopulations. Forward light scatter signal gives an indication of the particle size. The source of green autofluorescence in the microspores is not known. Data expressed on a log vs log display format, rather than a linear display format, presented the best microspore population

Table 1. Percentage embryo formation (means ± SE) for microspores/proembryos sorted 16 h and 72 h after culture initiation. Control (A) = non-sorted microspores/proembryos; control (B) = microspores/proembryos which passed through the flow cytometer but were not sorted. Data are based on 8 experiments.

<table>
<thead>
<tr>
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<th>Sorted</th>
<th>Control (A)</th>
<th>Control (B)</th>
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<tbody>
<tr>
<td>16 h</td>
<td>7.7 ± 1.76</td>
<td>1.4 ± 0.13</td>
<td>0.3 ± 0.02</td>
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<tr>
<td>72 h</td>
<td>13.7 ± 2.96</td>
<td>1.0 ± 0.19</td>
<td>1.1 ± 0.30</td>
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Figure 1. Highly heterogeneous microspore culture prior to (A) and subsequent (B) cell sorting at 16 h after culture initiation. Potentially embryogenic microspores were selected.