Regeneration of Plant Embryogenic Microspores Enriched with the WOLF G2® Gentle Cell Sorter

Introduction

Double haploid (DH) technology has become an integral component of commercial breeding programs in many agriculturally important crops[1–4]. The primary advantage of this technology is the production of homozygous haploid inducer lines in an accelerated manner[1,3]. The timeline differs greatly from the conventional breeding process, which requires approximately 6-10 generations of inbreeding to establish a desired cultivar[1]. DH methodology is used for both practical applications (e.g., breeding, mutagenesis, transformation, gene-editing) and fundamental research (e.g., biochemical, physiology, genomic studies). The process of DH production requires two steps: (1) haploid induction and (2) genome doubling, which is performed through chemical means[1,2,5,6].

Microspore culture is one methodology in plant biology used to generate DH plants, which are also defined as homozygous true breeding lines[1,5–10]. Microspores are immature pollen grains obtained from a donor plant, such as an F1 generation[1,5,6,8,9]. *Brassica napus*, also known as canola or rapeseed, is one of the main oil crops in the world and is also a model for routine DH development via microspore culture to accelerate the breeding of new cultivars[2]. The microspore, rather than following the gametophytic developmental route and fertilizing the egg cell to get seed, will develop into a haploid embryo[5,6,11]. Several factors can influence the response of the microspore including genotype, donor plant conditions, developmental stage of the pollen grain, pretreatments, media composition, and culture conditions[4–6]. Although *B. napus* is used as a model species for microspore culture, a low frequency of microspores responds to culture. An enrichment of the culture with responding microspores would increase the efficiency of the protocol.

The WOLF G2® Cell Sorter enriches populations of interest, such as microspores, using a gentle sorting mechanism (<2psi) with low shear stress, and supports cell viability by enabling the use of culture medium as sheath. Here, we demonstrate enrichment of *Brassica napus* microspores and the regeneration of embryogenic microspores into haploid plants.
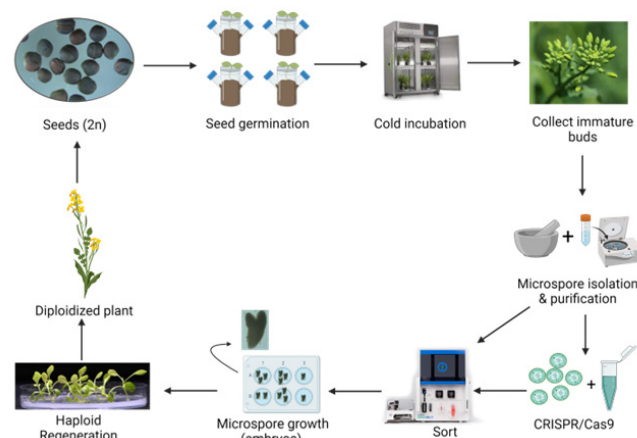

Figure 1. Microspore workflow for *Brassica napus* from seed to doubled haploid plant. Microspores are used for many aspects in plant biotechnology to generate DH plants that can be incorporated into breeding programs. Microspores can also be used in mutagenesis, transformation, or other genetic modification experiments with the resulting DH plants screened for the trait of interest, thereby a more rapid plant breeding methodology. This workflow was created with www.biorender.com and is based on protocols described in numerous *Brassica napus* publications[5,6,12].

Methods

Microspore Isolation and Induction

Brassica microspores were isolated from buds based on previous methods[5–7]. Briefly, donor plants were grown in environmentally controlled chambers set at 20/15°C (16 h light/ 8 h dark). Just prior to bolting, the temperature was reduced to 10/5°C while maintaining the same photoperiod. The buds were collected when the microspores reached the mid-late uninucleate stage. They were sterilized in 5.25% sodium hypochlorite for 15 minutes and washed three times in sterile distilled water. The *Brassica* buds were then crushed in half strength B5 medium with 13% sucrose (B5-13) to release the microspores. The microspores were purified further by washing in B5-13 media three times. The number of microspores was determined using a hemocytometer and the required amount of NLN-13 (NLN medium with 13% sucrose) was added to achieve a cell density of 105 microspores per mL. Microspores were dispensed into 100 x 15 mm petri plates at a volume of 10 mL/plate and incubated in the dark at 32°C. After 72 h, the plates were transferred to 24°C.

Microscope Images

Bright field images were taken on a Zeiss Axio Observer microscope pre- and post-sort to confirm cell intactness, enrichment of desired embryogenic cells, and division and development of the microspores to embryos.

Sorting *Brassica napus* with WOLF G2®

Freshly isolated *Brassica* microspores were brought to a cell density of approximately 4.0×10^4 microspores per mL in NLN-13 media. The cell mixture was then slowly filtered with a sterile wide-bore serological pipet through a 70 µm nylon sieve (Corning, cat# 352340) to remove cell clumps and large particles, which could clog the cartridge[13].

The WOLF G2 was primed and calibrated with sterile-filtered NLN-13 medium as sheath. Sterile 5.0 mL FACS tubes (Corning, cat# 352057) were placed in the tube holder intended for bulk cell sorts – three tubes corresponding to the three microfluidic sort channels named A, B, and C with B referring to the flow through and A and C for two distinct cell populations. When ready to sort, 1.5 mL of filtered microspores were transferred to a sterile 5.0 mL FACS tube. The microspores were identified on WOLF by comparing estimated cell size (FSC-H) and internal cell complexity (BSC-H) and then further separated using green autofluorescence (B525-H)(Figure 2)[14–16]. The gating strategy was as follows: all events expressing an FSC-H value above 1.0M and a BSC-H value above 100K are considered microspores; this is the parent gate (A). Following the parent gate is a child plot exclusive of singlet events to reduce coincidence in sorting, and it is gated to the parent (B). Third is a sub child plot displaying green positive (auto fluorescent) events solely inclusive of events from the singlets child gate (C). Lastly, (D) is a histogram representing the distribution of green auto fluorescent events in the population inclusive of only singlet events. The histogram is an alternative plot type to the scatter plot in C. Sorting for microspores herein was done using scatterplot C. Parameters and voltage gains of the sorts are listed in Table 1.

Table 1. Parameters and PMT detector gain settings for *Brassica napus* microspores on a WOLF G2 488/561 nm Cell Sorter. Fluorescence channel (FL) FL1 is dedicated to the 488 nm (blue) laser while FL2-FL5 channels are shared by both blue and yellow lasers, hence the "BY" nomenclature.

Parameter	Setting/Voltage (GAIN)
Laser Configuration	488 nm (blue), 561 nm (yellow)
Trigger Channel	FSC
FSC	10,000
FL1 (B525)	200 mV
FL2 (BY580)	200 mV
FL3 (BY620)	200 mV
FL4 (BY706)	200 mV
FL5 (BY760LP)	200 mV

NOTE: To prevent cell settling and cartridge clogging, it is suggested to pause the sort and gently invert the FACS tube every 30-45 minutes. Cell settling is indicated by a drop in the number of events per second and/or a visual observation at the bottom of the sample tube.

Embryo Development and Plant Regeneration[5]

After sorting, the petri plates with each respective population were placed back in the incubator in the dark stationary at 24°C. Two weeks after sorting, the petri plates were checked for embryo development. Once most of the developed embryos reached the cotyledonary stage, the petri plates containing the embryos were then moved to an orbital shaker set at 70 rpm in the light at 22°C. Once the embryos turned green, they were plated on a B5 agar medium for regeneration to plants.

Results

Viable *Brassica* microspores were identified through successive gating using estimated cell size (FSC-H) and internal cell complexity (BSC-H). Further separation of induced and non-induced was done visualizing the green autofluorescence signal (FL1; B525); gating strategy is described in Figure 2. Microscope images post sort show isolation of viable induced and non-induced microspore populations (Figure 3).

Three populations were collected through sorting, namely High-green (HG), mid-green (MG) and low-green (LG). The low and mid-green auto-fluorescent populations represent two types of induced (embryogenic) microspores and the high-green population correlates to the non-induced microspores (Figures 2, 3).

Sorted embryogenic microspores (MG, LG) were cultured and monitored for continued cell division and embryo formation. Embryos at various developmental stages were observed two weeks after sorting (Figure 4). The timeline of development matched with the control (unsorted) *Brassica* microspore cultures. The heterogeneous development of induced microspores is common in the control microspore cultures as well.

Embryos turned green three days after they were transferred to an orbital shaker in the light, which was earlier than the control *Brassica* microspore cultures, which took the standard 7 days. Plantlets regenerated normally after the embryos were plated on B5 solid medium (Figure 5).
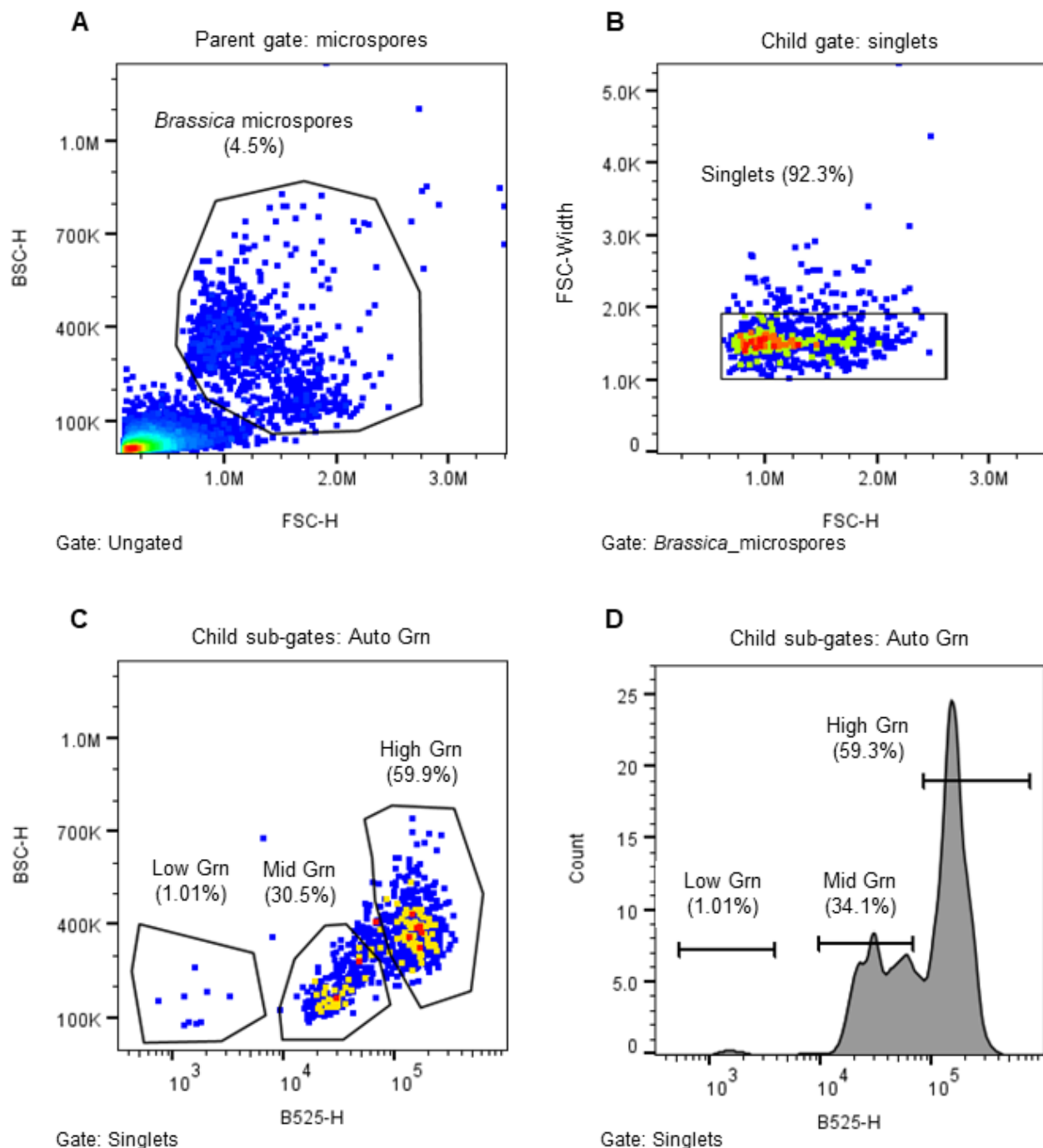
Figure 2. Sorting strategy for induced (embryogenic) *Brassica napus* microspores. Microspores were separated using estimated cell size (FSC-H), internal complexity/granularity (BSC-H), and green autofluorescence (FL1, B525-H) all excited by the 488nm laser. **A.** Parent gate (microspores), **B.** Child gate selecting for singlet events (gated to parent), **C.** Subsequent child gate selecting for green auto fluorescent positive events (gated to singlets), and **D.** A histogram representation of the green positive events within the child gate "singlets."
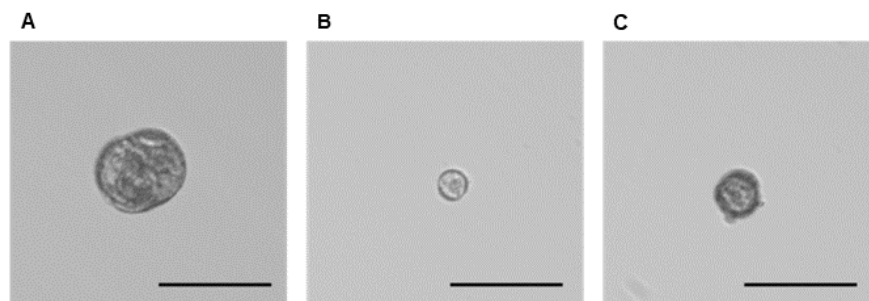
Figure 3. Phenotypes representative of three microspore populations sorted on WOLF G2. A. Low green autofluorescence, which correlates with the larger embryogenic microspore population (~1.0%), **B.** High green autofluorescence, which correlates with the non-embryogenic microspore population (~60.0%), and **C.** Mid-green autofluorescence, which correlates with an additional embryogenic microspore population (~30.0%). The scale bar on all three images is 50 μm.

Figure 4. Embryo development two weeks after sorting on WOLF G2. A. heart-stage embryo, **B.** torpedo-stage embryo, and **C.** cotyledonary-stage embryo. Images are representative of embryogenic microspores sorted from the low green autofluorescence population in Figure 1A. The scale bar on all three images is 200 μm.
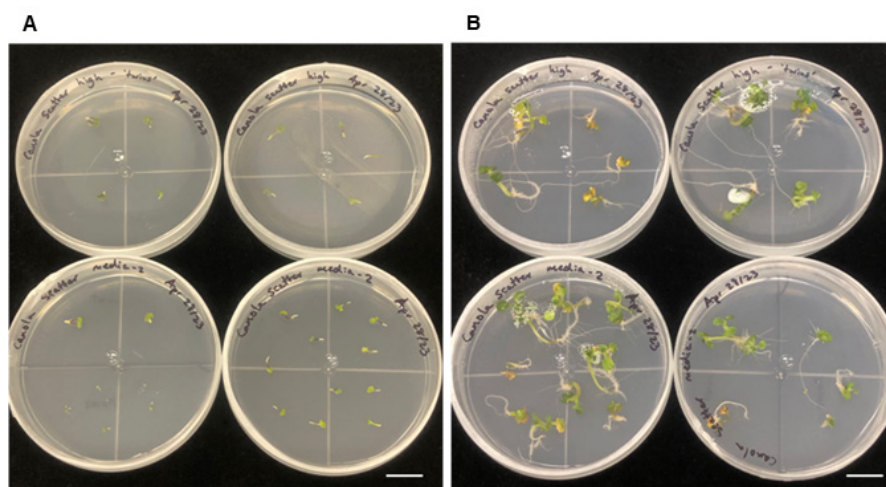

Figure 5. Freshly plated embryos and plantlet development from sorted embryogenic microspores. A. Freshly plated embryos, and **B.** plantlet development/regeneration two weeks after plating on solid medium. The scale bar on both images is 2.0 cm.

Conclusions

The production of homozygous true breeding lines is the goal of most commercial crop breeding systems worldwide[1–4]. Through traditional plant breeding processes, this can take 10+ years depending on the species[1,5]. Isolation of embryogenic microspores for DH production is one reliable method to create desirable cultivars in an accelerated manner[1,2,4–6]. Many canola (*Brassica napus*) breeding programs utilize this workflow to produce improved cultivars that meet canola oil standards (Figure 1). The addition of gentle cell sorting empowers scientists with a tool to select for embryogenic microspores from a heterogenous population, which further increases the efficiency of the protocol. Embryogenic microspores are desirable as they can be used in a range of workflows in both practical applications, such as DH production, and fundamental research. The WOLF G2 successfully sorted three populations of *Brassica napus* microspores, where the two sorted embryogenic populations successfully regenerated into plants (Figures 2-5).

For more information, visit nanocellect.com or email info@nanocellect.com

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