EMBRYOGENESIS FROM MICROSPORES OF EMBRYOGENIC AND NON-EMBRYOGENIC LINES OF BRASSICA NAPUS

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INTRODUCTION

The frequency of embryo production from isolated microspores of Brassica napus has been greatly improved from the time it was first reported (Lichter 1982). To obtain a highly efficient system, it is necessary to screen genotypes and carefully select buds (Chuong et al. 1987; Pechan and Keller 1988), correctly stage microspores (Kott et al. 1987a and 1987b; Fan et al. 1988; Fife et al. 1991), and optimize donor plant growth conditions (Keller et al. 1987) and in vitro culture conditions (Gland et al. 1988; Huang et al. 1990). There is a great variation in embryo production from different genotypes. Some genotypes yield large numbers of embryos whereas others, particularly winter rape varieties, show different degrees of recalcitrance producing very few or no embryos.

The change from pollen development to embryo development requires a major change in cell morphogenesis which must invoke the reorganization of microtubules (MTs), the morphogenic elements of the cell. In this study, MT reorganization during early induction is used as a marker of embryogenic potential. The MT organization during prophase of the highly embryogenic cv. Topas is compared to that of the recalcitrant line 0025. Furthermore, the possibility of inducing recalcitrant lines and incorrectly staged microspores to develop into normal embryos with the help of nurse cultures of responsive lines is examined.

MATERIALS AND METHODS

Microspores from individual buds of B. napus Topas and 0025 were isolated and cultured as described by Fife et al. (1991). For nurse culture experiments, pre-washed 30 mm Millicell-CM inserts (Millipore) were placed inside 20x60 mm plastic petri dishes (Lab Tek, Nunc). The recalcitrant microspore culture (0.3 ml) was placed inside the inserts and the nurse culture (1.5 ml) was placed in the petri dish, both at a microspore density of $2x10^4$ cells/ml. A cellulose ester membrane, pore size 0.45 um, separated the two cultures.

MT organization during prophase was analyzed by using a double staining procedure on fixed microspores. Chromatin was stained with Hoechst 33258 and the MTs were labelled using indirect immunofluorescence with anti-tubulin antibody

(Simmonds et al. 1985; Simmonds & Setterfield 1986). Prophases were easily identified by the characteristic condensation of chromatin (Figs. 1b-4b). In order to examine MT organization at prophase of microspores in different developmental pathways the following microspore preparations of Topas and 0025 were used:

1. Pollen Development - first pollen mitosis in freshly isolated untreated microspores
2. Embryo Development - isolated uninucleate microspores, first division after 8-10 h of culture at 32.5°C
3. Non-induced microspores - isolated uninucleate microspores, first division after 12-16 h of culture at 25°C

RESULTS AND DISCUSSION

PPB microtubules associated with embryo induction

Microtubule organization during prophase can be indicative of future changes in differentiation because the preprophase band (PPB) predicts future planes of division thus determining morphological patterns. Therefore MT organization during prophase was examined in the first pollen division, and the first division of induced and non-induced cultured uninucleate microspores of Topas and 0025.

The MT organization in late uninucleate microspores and prophases of the first pollen mitosis is similar for Topas and 0025 lines. The MTs of late uninucleate microspores are associated with the surface of the nuclear envelope. The nucleus in prophase is laterally located, adjacent to the centre of one of the three lobes. The MTs during prophase of the first pollen mitosis radiate from all sides of the nucleus except from the area closely juxtaposed to the lobe (Fig. 1). Cortical MTs are not present at this stage. Similar MT organization is found in non-induced cultured microspores of both Topas and 0025.

During induction of late uninucleate microspores of Topas, the pattern of MT organization changes. The MT strands radiating from the nuclear envelope increase in density and length. The cells enter prophase after 8-12 h of culture at 32.5°C. Typical PPBs are found in the cell cortex at prophase, often in an equatorial plane (Figs. 2 and 3) thus predicting the plane of the future cell plate. Temperature induced cells divide symmetrically and go on to form embryos. Non-induced cells and first pollen divisions are asymmetric and proceed along the pollen development pathway. The PPB is the first distinct structural change in microspores to indicate a reprogrammed developmental pathway. Further confirmation of the PPB signalling the onset of embryogenesis in microspores is found in the non-embryogenic line 0025. Normal PPBs are not formed in 0025 microspores cultured at the Topas induction temperature of 32.5°C. Although most of the cells show prophase MTs similar to those of the first pollen mitosis, a small number of cells show a change in MT organization.

Cortical MTs in more or less parallel arrays appear in 0025 microspores (Fig. 4). However, this is only an attempt to form PPBs as these MTs do not organize to form normal PPBs.

Use of nurse cultures to induce recalcitrant lines and circumvent incorrectly staged buds

The results from six experiments with several different plants yielded no embryos from the recalcitrant line 0025. The plant donor conditions, staging and culturing were the same as those optimized for Topas. It was possible that 0025 is totally recalcitrant to microspore embryogenesis but it was more likely that different pretreatment and/or culturing conditions are required (Gland et al. 1988). The small number of temperature treated 0025 microspores which showed an attempt to form PPBs may have been the ones with embryogenic potential. However, the frequency of these microspores may have been too low to give rise to embryos. It has been shown that under optimal conditions of culture the minimum microspore density of 3000/ml is required for embryogenesis of Topas (Huang et al. 1990). At lower densities embryos do not form although microspores with embryogenic potential are present. Therefore nurse cultures of Topas were used to increase the density of embryogenic microspores. Twenty eight embryos were generated from a total of three experiments, seven nurse cultures and 80,000 microspores of 0025.

Nurse cultures were also tested to examine the possibility of eliminating abnormal embryo morphology from microspores cultures which had been staged incorrectly. Similar sizes of buds, petals and anthers are found in buds carrying class III and IV microspores, but the difference in microspore stage and frequency of embryogenesis is profound (Fife et al. 1991). Class III microspores include late uninucleate and 1-87% binucleate microspores and normal embryos are produced at a frequency of 21.4 $\pm$ 3.0%. Class IV contains more than 87% binucleates of which the majority is early binucleate, and produces embryos at a frequency of 3.0 $\pm$ 1.7% which are often abnormal. The experiment shown in Table 1 and Figs. 5-8 demonstrates that when Class IV microspores are grown on nurse cultures of Class III a higher frequency of embryogenesis is obtained but more important, the embryos develop normally [compare Fig. 6 (Class IV, no nurse) with Fig. 8 (Class IV, with nurse)]. Furthermore, the frequency of embryo production of Class III microspores is reduced from 18.3% to 13.5% when cultured in the presence of Class IV. This supports the suggestion of Kott et al. (1987) that binucleate microspores may release toxic substances which inhibit embryogenesis. It is interesting that inhibitory effects of Class IV were evident on the Class III nurse culture when the microspores were cultured at a ratio of 1:5 (ClassIV:ClassIII). With ratios higher in Class IV microspores, greater embryo abnormalities and lower frequencies of embryogenesis would be expected. As buds staged for Class III or IV microspores have very similar morphologies (Fife et al. 1991) a mixture of the two bud types would be common in multiple bud preparations which would result in a decrease in embryo frequency and

increase in embryo abnormalities. This suggests that when normal embryos are required for haploid plant production it is preferable to err on the side of smaller buds for microspore culture. The frequency of embryogenesis may be reduced but the presence of toxins from binucleate microspores which produce abnormal embryos will be eliminated.

Table 1. Culture of Class IV Topas microspores on Class III nurse. Microspore density = 2 x 10^4/ml.

Class	No. of Microspores	% Embryogenesis
III (control)	36,000	18.3[a] (17.7-18.6)
IV (control)	36,000	2.0 (1.9-2.1)
III (nurse)	30,000	13.5 (12.9-14.1)
IV (insert)	6,000	3.7 (3.5-3.8)

a - The figure is the mean of three samples and range is shown in brackets.

In conclusion, MT organization at prophase has indicated that a low frequency of microspores of line 0025 may have embryo potential. This was confirmed by the use of nurse cultures of Topas to induce embryogenesis of 0025. Nurse cultures of Topas were also used to induce normal development of abnormal embryos which are produced from buds containing more than 87% binucleate microspores. Nurse cultures of Topas may be helpful for embryogenesis and haploid plant production in recalcitrant species where a large number of embryos is not required. The nurse cultures may also be useful as a first step in screening recalcitrant species of Brassica as optimal staging is not necessary and even very low frequencies of microspores with embryogenic potential can be expressed. The lines showing embryogenic potential could then undergo systematic modification for optimal microspore isolation and culture. This approach would eliminate labour intensive and possibly wasteful work on highly recalcitrant species, postponing work on these species until the technology is more advanced.

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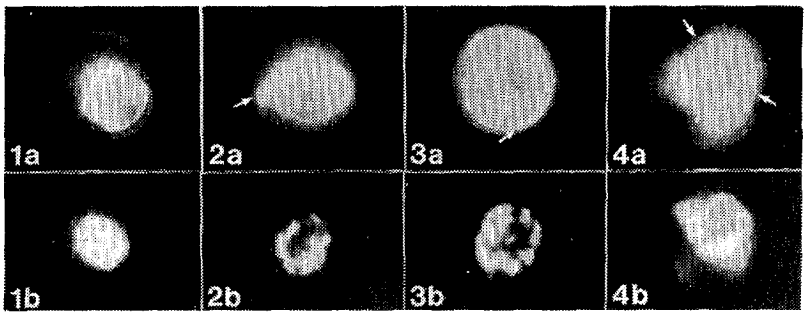
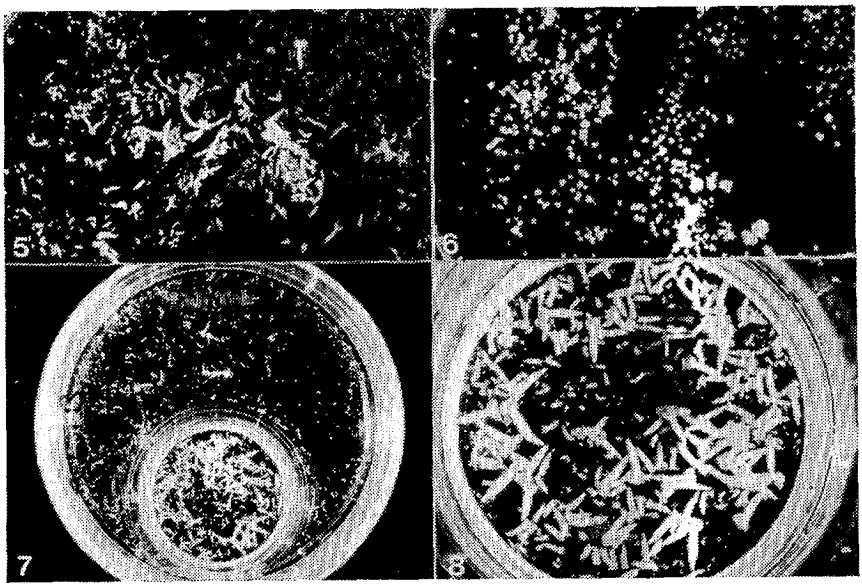


Figs. 1-4. Isolated microspores of Topas (Figs. 1-3) and 0025 (Fig. 4) fixed and labelled for microtubules by immunofluorescence (Figs. 1a-4a) and stained for DNA with Hoechst 33258 (Figs. 1b-4b). Fig. 1. Prophase of the first pollen mitosis showing MTs radiating from the nuclear surface. Figs. 2 and 3. Prophase of the first division during induction of Topas showing an equatorial PPB perpendicular to the focal plane (Fig. 2, arrow) and a PPB in the plane of focus (Fig. 3, arrow). Fig. 4. Prophase of 32.5°C treated 0025 microspore showing parallel MT arrangement in the cell cortex (arrow) but no PPB.



Figs. 5-8. Culture of Topas microspores Class IV on nurse cultures of Class III as outlined in Table 1. Fig. 5. Control Class III showing cotyledonary embryos. Fig. 6. Control Class IV showing abnormal globular and multilobed embryos. Fig. 7. Nurse culture of Class III in petri dish and Class IV in insert. Fig. 8. Enlarged insert of Fig. 7 showing normal embryos of Class IV at the cotyledonary stage.