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Significance of preprophase bands of microtubules in the induction of microspore embryogenesis of *Brassica napus*

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Abstract. Microspores of *Brassica napus* L. cv. Topas, undergo embryogenesis when cultured at 32.5 °C for the first 18–24 h and then at 25 °C. The first division in heat-treated microspores is a symmetric division in contrast to the asymmetric division found after the first pollen mitosis in-planta or in microspores cultured continuously at 25 °C. This asymmetric division is unique in higher plants as it results in daughter cells separated by a non-consolidated wall. The cytoskeleton has an important role in such morphological changes. We examined microtubule (MT) organization during the first 24 h of heat induction in the embryogenic *B. napus* cv. Topas and the non-embryogenic *B. napus* breeding line 0025. Preprophase bands (PPBs) of MTs appeared in cv. Topas microspores in late uninucleate microspores and in prophase figures after 4–8 h of heat treatment. However, more than 60% of the PPBs were not continuous bands. In contrast, PPBs were never observed in pollen mitosis; MT strands radiated from the surface of the nuclear envelope throughout microspore maturation to the end of prophase of pollen mitosis I, during in-planta development and in microspores cultured at 25 °C. Following 24 h of heat treatment, over 95% of the microspores appeared to have divided symmetrically as indicated by the similar size of the daughter nuclei, but only 7–16% of the microspores eventually formed embryos. Discontinuous walls were observed in more than 50% of the divisions and it is probable that the discontinuous PPBs gave rise to such wall abnormalities which may then obstruct embryo development. Preprophase bands were not formed in heat-treated microspores of the non-embryogenic line 0025 and the ensuing divisions showed discontinuous walls. It is concluded that the appearance of PPBs in heat-induced microspores marks sporophytic development and that continuous PPBs are required for cell wall consolidation and embryogenesis. It follows that induced structures with two equally condensed nuclei, do not necessarily denote symmetric divisions.

Key words: Brassica (microspore embryogenesis) – Cell wall – Embryogenesis – Microspore (division symmetry) – Microtubule – Preprophase band

Introduction

The highly androgenic *Brassica napus* L. cv. Topas has been used to study the mechanisms involved in changing the developmental fate of microspores from gametogenesis to sporogenesis (Fan et al. 1988; Zaki and Dickinson 1990, 1991; Hause et al. 1993; Telmer et al. 1993, 1995; Cordewener et al. 1995; Zhao et al. 1996a). During pollen development, microspores divide asymmetrically to produce a vegetative cell and a generative cell, two morphologically distinct daughter cells (Telmer et al. 1993). The latter cell divides again to produce two sperm cells. Heat-treated microspores undergo a number of morphological changes that culminate in a symmetric division with daughter cells similar in size and appearance (Zaki and Dickinson 1990; Telmer et al. 1993, 1995). A preliminary investigation indicated that a change in microtubule (MT) organization during heat induction of microspores, namely the appearance of a preprophase band (PPB) of MTs, may determine division symmetry (Simmonds et al. 1991; Simmonds 1994). A PPB is a band of cortical MTs which appears transiently prior to prophase and disappears before metaphase in most higher-plant cells. The cell plate makes contact with the parental wall at precisely the site previously occupied by the PPB, thus indicating that this site is somehow marked (Picket-Heaps and Northcote 1966; Gunning 1982; Baskin and Cande 1990). This site

Abbreviations: DIC = differential interference contrast; LU = late uninucleate; MT = microtubule; MU = mid-uninucleate; NC = nuclear-cortical; PPB = preprophase band; UV = uninucleate vacuolate

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is also significant in that it harbours putative 'factors' which promote maturation and consolidation of the newly formed cell wall (Mineyuki and Gunning, 1990). The first pollen mitosis in microspores lacks a PPB (Van Lammeren et al. 1985; Terasaka and Niitsu 1990; Simmonds et al. 1991) and results in a non-consolidated cell plate. An unstable cell plate is undoubtedly an important feature in this division because the generative cell is destined to be mobile within the vegetative cell.

It has been suggested that the change in division symmetry may be sufficient to induce embryogenesis (Zaki and Dickinson 1990). However, as 90–98% of the microspores undergo a symmetric division but only 10–30% regenerate embryos, other factors must be involved in determination of embryogenesis (Telmer et al. 1992, 1995). This study describes the changes in microtubule organization of heat-treated microspores and shows that in *B. napus* cv. Topas, two types of PPBs are induced; a majority of PPBs are discontinuous and only a minority are normal continuous bands of MTs. Furthermore, the data indicate that the continuous PPBs lead to wall consolidation, a critical step in establishing a bicellular structure, the initial event of a multicellular structure, which then leads to embryogenesis.

Materials and methods

Donor plants and microspore cultures. The plant material used was the highly embryogenic *B. napus* cv. Topas, line 4079 and the *B. napus* breeding line 0025 (kindly provided by Allelix Inc.) which was not embryogenic under the same induction conditions. Growth conditions for donor plants and the procedures for microspore staging and isolation were carried out as described previously (Telmer et al. 1992). Microspores were cultured at a cell density of 2×10^4 ml^{-1} NLN-13 (NLN medium with 13% sucrose and without potato extract, Lichter 1982) in 600-μL volumes, in Petri plates (35 mm diameter, 10 mm deep). The plates were incubated in humid chambers at 25 °C or 32.5 °C in darkness. Following the 24-h heat treatment (32.5 °C), plates were cultured at 25 °C in darkness.

Labelling for microtubules and DNA. Freshly isolated microspores or microspores cultured for 4, 8, 12, 16, 24 or 48 h were fixed for 1 h in 3% paraformaldehyde by mixing equal volumes of microspores in NLN-13 medium and 6% paraformaldehyde in MTB1 [0.1 M mannitol, 25 mM phosphate buffer (pH 7.0), 10% dimethyl sulphoxide, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} leupeptin] containing 0.02% Nonidet P-40. Following fixation, the paraformaldehyde was diluted to 1.5% by the addition of an equal volume of MTB1, the microspores were pelleted by centrifugation (5 min, 100 *g*), washed two more times in MTB1 and resuspended in enzyme buffer [0.1 M mannitol, 25 mM phosphate buffer (pH 7.0), 5 mM ethyleneglycol-bis-(β-amino ethylether)-N,N,N′,N′-tetra-acetic acid, 1 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} leupeptin) containing 1% β-glucuronidase (# G 7770; Sigma Chemical Co.). Following a 15-min incubation the microspores were washed two times in MTB1, as above, and each sample was resuspended in a very small volume of MTB1 (approx. 70 μL). A drop of the suspension was spread on a polylysine-coated coverslip and allowed to settle for 5 min after which time the samples were extracted for 20 min in 1% Nonidet P-40 in MTB1, washed for 5 min twice in MTB1, and three times in PBS for a total time of 15 min. Monoclonal rat anti-yeast tubulin (MAS 078, clone YOL 1/34; Cedarlane Laboratories, Hornby, Ont., Canada) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat immunoglobulin IgG (Sigma) were the primary and secondary antibodies, respectively. The samples were processed for antibody labelling and Hoechst 33258 staining and were mounted in 50% glycerol in PBS containing 0.1% *p*-phenylenediamine as previously described (Simmonds and Setterfield 1986). The preparations were examined using a Zeiss Photomicroscope III equipped with epifluorescence optics or a Zeiss Axiophot equipped with epifluorescence and differential interference contrast (DIC) optics and the appropriate filters. Ilford XP2 400 chromogenic black and white film was used for photography.

Microtubule organization during microspore development and culture at 32.5 °C and 25 °C. For analysis of MT organization of in-planta microspore development, microspores were isolated from buds with microspores stages ranging from mid-uninucleate (MU) to early bicellular. Each stage was analysed in at least six experiments.

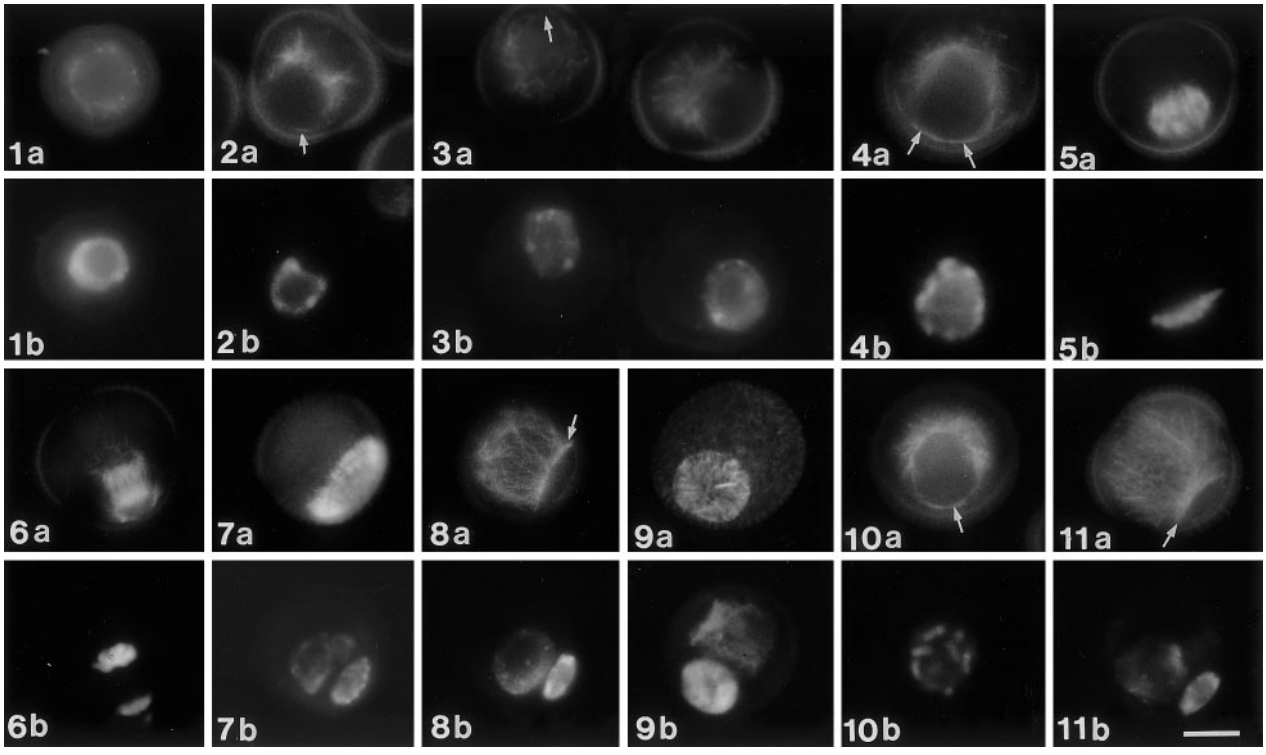
Microspore populations used in culture experiments consisted of a majority of late uninucleate (LU), a minority of uninucleate vacuolate (UV) microspores and 0–3% bicellular and mitotic structures. Analysis of MT organization in microspores following 4, 8, 12, 16, 24 or 48 h of culture at 32.5 °C and 25 °C was conducted in at least ten experiments for *B. napus* cv. Topas and in four experiments for the non-embryogenic line 0025.

Continuity of PPBs, wall integrity and frequency of embryogenesis. Three experiments were conducted to analyse PPB and cross-wall continuity. After 8 h of culture, PPB continuity was evaluated by fluorescence microscopic examination of the structures through all focal planes. Binucleate structures were analysed after 24 h of culture by scoring continuity of fluorescence at the newly formed cross-wall and cross-wall integrity using DIC optics. Following 3 weeks of culture, embryos were counted in three Petri plates for each experiment, averaged and expressed as a percentage of viable microspores at the time of plating. Intact cells with cytoplasmic contents were scored as viable immediately after plating. After 24 h of heat induction, only swollen microspores were considered to be viable.

Results

Microtubule organization of in-planta microspores: uninucleate, mitotic and early bicellular developmental stages. Throughout microsporogenesis, up to and including prophase, MT labelling is concentrated at the nuclear envelope and from this region, strands of MTs radiate into the cytoplasm. During early to mid-uninucleate (MU) development, faint perinuclear fluorescence is associated with a nucleus positioned near the centre of the cell (Fig. 1). During the MU stage, small vacuoles fuse to form larger ones which also merge and enlarge into one vacuole which marks the beginning of the UV stage. The UV microspore is spherical and the wall is divided into three lobes by three colpi or furrows which do not converge at the poles. The nucleus, displaced by the expanding vacuole, is positioned close to the plasma membrane, at the centre of one of the three lobes, subsequently referred to as the 'nuclear lobe'; MT strands, more abundant at this stage, radiate from the nuclear envelope and skirt around the vacuole (Fig. 2). Following the UV stage, the vacuole fragments. The LU stage of microspore development is characterized by numerous small vacuoles situated in the cell cortex opposite the nucleus which remains appressed to the nuclear lobe (Telmer et al. 1992), and numerous MT strands radiate from all sides of the nuclear envelope with the exception of the surface appressed to the

plasma membrane (Fig. 3). An asymmetric first pollen mitosis follows and shows all mitotic figures positioned at the periphery of the microspore. The MT organization at prophase (Fig. 4) appears similar to that of the LU stage (Fig. 3), with one difference; intense MT labelling is present within the thin layer of cytoplasm between the nuclear membrane and the plasma membrane (Fig. 4, arrows), subsequently referred to as the 'nuclear-cortical' (NC) zone. Frequently, the labelling extends beyond the NC zone along the cortex of the nuclear lobe (Fig. 4). Faint labelling could be discerned at the NC zone at the UV (Fig. 2, arrow) and LU (Fig. 3, arrow) stages. The metaphase spindle is positioned at the centre of the 'nuclear lobe', in the



Figs. 1–11. Microtubule organization during *B. napus* cv. Topas microspore development from MU to bicellular stages. The cells are simultaneously labelled for MTs by immunofluorescence (Figs. 1a–11a) and DNA by Hoechst 33258 (Figs. 1b–11b). Scale bar = 10 μm

Fig. 1a,b. Mid-uninucleate microspore shows perinuclear fluorescence and a few radiating strands of MTs (**a**). The nucleus is situated near the centre of the cell (**b**)

Fig. 2a,b. Uninucleate vacuolate microspore shows radiating MTs at the nuclear surface and extending around the vacuole (**a**); there is faint fluorescence at the NC zone (**a**, *arrow*). The nucleus is positioned at the edge of the cell (**b**); note the nuclear depression exerted by the vacuole

Fig. 3a,b. Two LU microspores at different orientations show a cross-section, perpendicular to the axis of the microspore (*right*) and an oblique section just below the nuclear lobe (*left*) (**a**); the microspore on the right shows strands of MTs radiating in all directions from the nuclear surface; the optical focal plane near the nuclear surface of the cell on the left shows faint fluorescence at the NC zone where the plasma membrane has pulled away from the wall (**a**, arrow). The nucleus is at the edge of each microspore (**b**)

Fig. 4a,b. Microspore at prophase shows MT strands radiating from the nuclear surface and intense fluorescence at the NC zone (*arrows*) and the cortex of the nuclear lobe (**a**). Chromatin condensation is apparent (**b**)

Fig. 5a,b. The metaphase spindle appears appressed to the inner face of the wall (**a**). The equatorial plate is parallel to the cell wall (**b**)

Fig. 6a,b. Anaphase/telophase transition, at the edge of the cell, shows an early MT phragmoplast and the remains of polar MTs (**a**), and differential condensation of chromatin as indicated by the difference in nuclear size (**b**). The smaller of the two nuclei is normally brighter, but objects in the light path can deflect the fluorescence

Fig. 7a,b. A microspore at telophase shows the acentric location of the MT phragmoplast (**a**). The nucleus of the generative cell is condensed whereas the vegetative cell nucleus is pleomorphic and has dispersed chromatin (**b**)

Fig. 8a,b. The optical plane of focus at the surface of a bicellular pollen grain shows bright MT labelling at the cross-wall between the two daughter cells (*arrow*) and cortical MTs in the vegetative cell (**a**). The optical cross-section shows differentially condensed nuclei (**b**)

Fig. 9a,b. The optical plane of focus coincides with the plane of the cross-wall of a bicellular pollen grain and shows MT strands converging from the parental wall (**a**). The nuclei are differentially condensed (**b**)

Fig. 10a,b. A microspore in prophase, after 8 h of culture at 25 °C, shows MTs radiating from the nuclear envelope and bright fluorescence at the NC zone (*arrow*; **a**). Chromosomes are condensed (**b**)

Fig. 11a,b. A bicellular pollen grain, after 24 h of culture at 25 °C, shows cortical MTs in the vegetative cell and bright fluorescence at the cross-wall (*arrow*; **a**). The nuclei are differentially condensed (**b**)

equatorial plane, perpendicular to the microspore wall (Fig. 5). Differential condensation of the generative and the vegetative chromatin is frequently apparent during the anaphase/telophase transition (Fig. 6a,b). During early telophase, the phragmoplast forms at the site of the previous equatorial plate and expands centrifugally in this plane but as it matures, it curves around the generative cell thus forming a lens-shaped wall (Fig. 7). This asymmetric division results in a large vegetative cell and a large nucleus with dispersed chromatin, and a small generative cell with a small, highly condensed nucleus (Fig. 8b). Bright MT labelling is present at the cross-wall separating the daughter cells (Fig. 8a, arrow) and from this region, cortical MT strands appear to extend across the vegetative cell. The cortical MT arrays at the cross-wall are arranged in a radial fashion converging from the junction of the cross-wall with the parental wall (Fig. 9). It was not possible to determine whether this MT array was present in the cortex of the vegetative cell, the generative cell or both cells.

Microtubule organization in cultured microspores of B. napus cv. Topas

Culture at 25 °C. Microspores cultured at 25 °C continued pollen development and displayed MT arrangements similar to that of the in-vivo organization (Figs. 10, 11).

Culture at 32.5 °C. Microspores cultured at 32.5 °C for 24 h were induced to undergo embryogenesis. During the 24-h culture period, 92–99% of the microspores undergo 'symmetric division' which produces two equally condensed daughter nuclei (Table 1). This indicates that LU and UV microspores, isolated and cultured at G2, S and possibly G1 phases of the cell cycle (Binarova et al. 1993) can be induced to divide symmetrically.

After 4–8 h of heat treatment, changes in MT organization were evident in microspores close to mitosis. The previously dominant perinuclear fluorescence was diminished and a ring of parallel cortical MTs, the PPB, was formed (Figs. 12–14). Cortical MT formation was not observed during this culture period. Wide and narrow PPBs appear in LU (Figs. 12, 13). During prophase, the PPBs are usually narrow and the majority of the prophase figures show a central nucleus (Fig. 14). The plane of the PPB is oblique to the plane of the polar axis (the longitudinal plane; Fig. 12) and may deviate from the longitudinal plane by more than 45° (Fig. 14c, cell I). An optical transverse section shows that the PPB passes through the NC zone (Fig. 13b, two arrows). The PPB was never observed to traverse through the polar regions. Prophase figures without PPBs were observed very rarely. Microspores showing random arrays of cortical MTs, diminished perinuclear fluorescence and cytoplasmic MTs were observed after 12 h of culture (Fig. 15). These cortical microtubules may participate in the formation of a cellulosic wall as described by Zaki and Dickinson (1990).

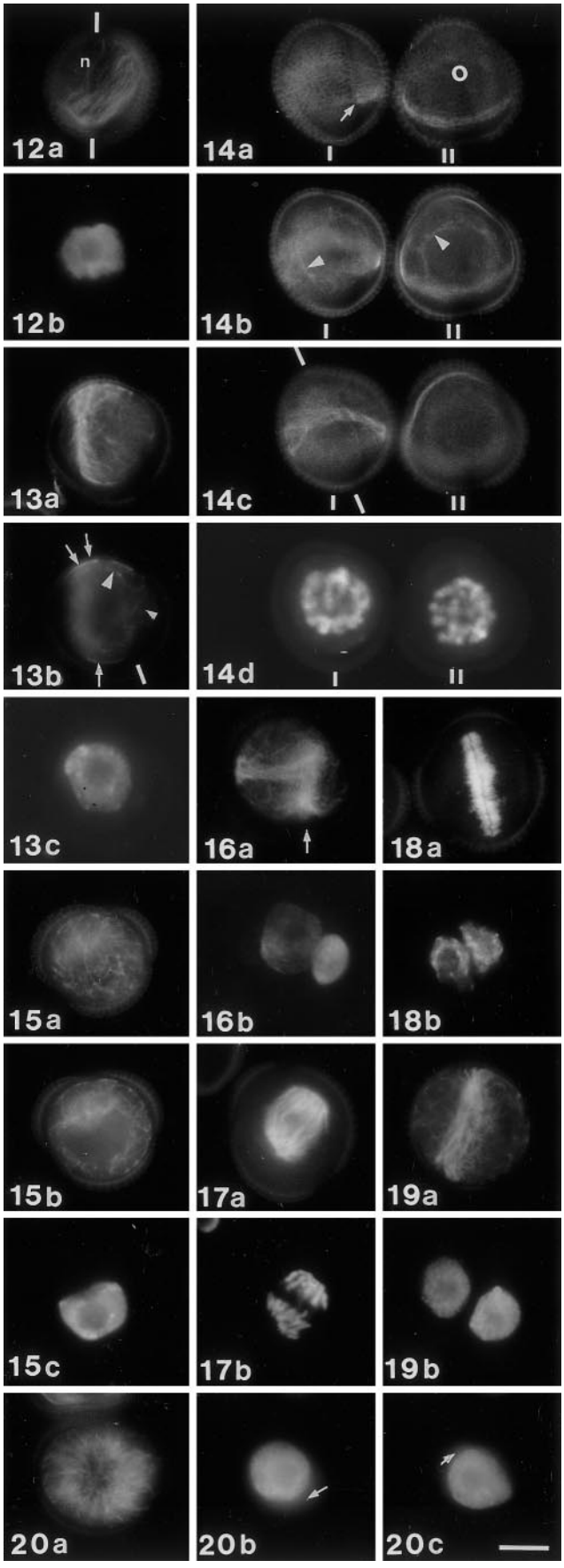
This report is focused on microspore embryogenesis from uninucleate microspores but since embryogenesis can be induced from early bicellular pollen (Zhao et al. 1996a), it is noteworthy that PPBs have been observed in vegetative cells of bicellular pollen grains (Fig. 16).

Numerous mitotic figures were observed at 8–12 h of culture. Metaphase and anaphase figures are located near the centre of the microspore with the spindle axes oriented in a transverse plane (Fig. 17a). At telophase, the phragmoplast spans across the centre of the microspore dividing the cell into two fairly equal halves (Fig. 18a). The chromatin of the daughter nuclei is equally condensed at anaphase and telophase (Figs. 17b, 18b). Such divisions produced two daughter cells similar in size and nuclear appearance (Fig. 19) with an equal distribution of organelles (Telmer et al. 1993). Randomly oriented cortical MTs appeared in the daughter cells and intense fluorescence was observed in the transverse sections of the new cross-wall (Fig. 19a). Optical sections of the cross-wall in face view revealed that the cortical MTs were radially arranged (Fig. 20a) as they were after the first pollen mitosis (Fig. 9a).

Discontinuous PPBs and cross-walls. The PPBs were not always continuous as those described above. One of the two cells at prophase in Fig. 14 (cell I) shows the PPB terminating at a furrow (Fig. 14a, arrow). Discontinuous PPBs occurred at a frequency of 60–75% (Table 1). Discontinuous cross-walls were also a frequent occurrence (Figs. 21, 22). Intense MT fluorescence is associated with the cross-wall after the first microspore division (Fig. 19a). Discontinuous fluorescence at the cross-wall, as shown at two optical focal planes in Figs. 21a and 21b, is a good indication that the cross-wall is also discontinuous. This can be observed more clearly in the structure shown in Fig. 22 where the cross-wall-associated fluorescence terminates before reaching the plasma membrane and shows continuous cytoplasm between the 'daughter cells' in the region lacking fluorescence (Fig. 22a, arrow). Differential interference contrast (DIC) microscopy of the same structure illus-

Table 1. Integrity of preprophase bands (PPBs) and cross-walls formed during heat-induction of *Brassica napus* cv. Topas microspores. Three samples were scored from each of three experiments. PPBs were scored after 8 h of heat induction, walls and 'symmetric divisions' (daughter nuclei of similar size) were scored after 24 h of heat induction, and embryogenesis was scored after 3 weeks of culture. Cross-walls were examined with both DIC and fluorescence optics as described in *Materials and methods*. For each experiment, a minimum of 45 PPBs, 80 cross-walls and 300 divisions were scored. All embryos in three Petri dishes were counted and expressed as percent of viable microspores plated where viable microspores frequency was 87–92%. After 24 h of culture, microspore viability was reduced to 75–83% and over 95% of the viable microspores had divided.

% Continuous PPBs	% Complete cross-walls	% 'Symmetric divisions'	% Embryogenesis
25	32	99	7.4
40	48	98	15.8
30	40	92	12.1



Figs. 12–20. *Brassica napus* cv. Topas microspores, after 8–24 h of culture at 32.5 °C, simultaneously labelled for MTs by immunofluorescence (Figs. 12a, 13a,b, 14a–c, 15a,b, 16a–20a) and DNA by Hoechst 33258 (Figs. 12b, 13c, 14d, 15c, 16b–19b, 20b,c). Scale bar = 10 μm

Fig. 12a,b. An LU microspore, after 8 h of culture, shows a wide PPB crossing the furrow (position indicated by *lines*); the plane of the PPB is oblique to the plane of the polar axis (*lines*; **a**). The nucleus (*n* in **a**) is positioned peripherally within the lobe opposite the furrow traversed by the PPB (**b**)

Fig. 13a–c. An LU microspore showing a narrow PPB at the surface of the cell (**a**) and at an optical transverse section of the cell (**b**) where the 'edge-on' view shows the PPB to coincide with the NC zone (*two arrows*) on one side of the cell, and on the other side (*one arrow*) to be close to the furrow (*line*). Perinuclear fluorescence in (**b**) is very faint (*arrowheads*). The nucleus is situated at the cell periphery (**c**)

Fig. 14a–d. Two microspores in prophase, after 8 h of culture. Preprophase bands are shown at the two surface optical planes of focus (**a, c**) and at the optical cross-section (**b**). A continuous PPB is present in Cell II at both surfaces and through the cross-section while the PPB of Cell I is discontinuous, terminating at a furrow (**a**, *arrow*). The polar axis of Cell I is in the plane of the micrograph (its orientation is noted by lines in **c**) and the plane of the PPB is almost transverse. In Cell II, the polar axis is almost perpendicular to the micrograph (**a**, the pole is marked *O*), and the plane of the PPB is oblique to the polar axis. The optical cross-sections show faint perinuclear fluorescence (**b**, *arrowheads*) and centrally positioned nuclei with condensed chromosomes (**d**)

Fig. 15a–c. A uninucleate microspore, after 12 h of culture, shows cortical (**a**) and cytoplasmic (**b**) MTs, and faint perinuclear fluorescence (**b**). The nucleus is relocated to a more central position of the microspore (**c**)

Fig. 16a,b. A bicellular structure after 12 h of culture shows a PPB in the vegetative cell, bright MT fluorescence at the cross-wall between the two cells (*arrow*; **a**) and differentially condensed nuclei (**b**)

Fig. 17a,b. A microspore in anaphase, after 12 h of culture, shows a spindle positioned close to the centre of the cell (**a**) and chromosome separation (**b**)

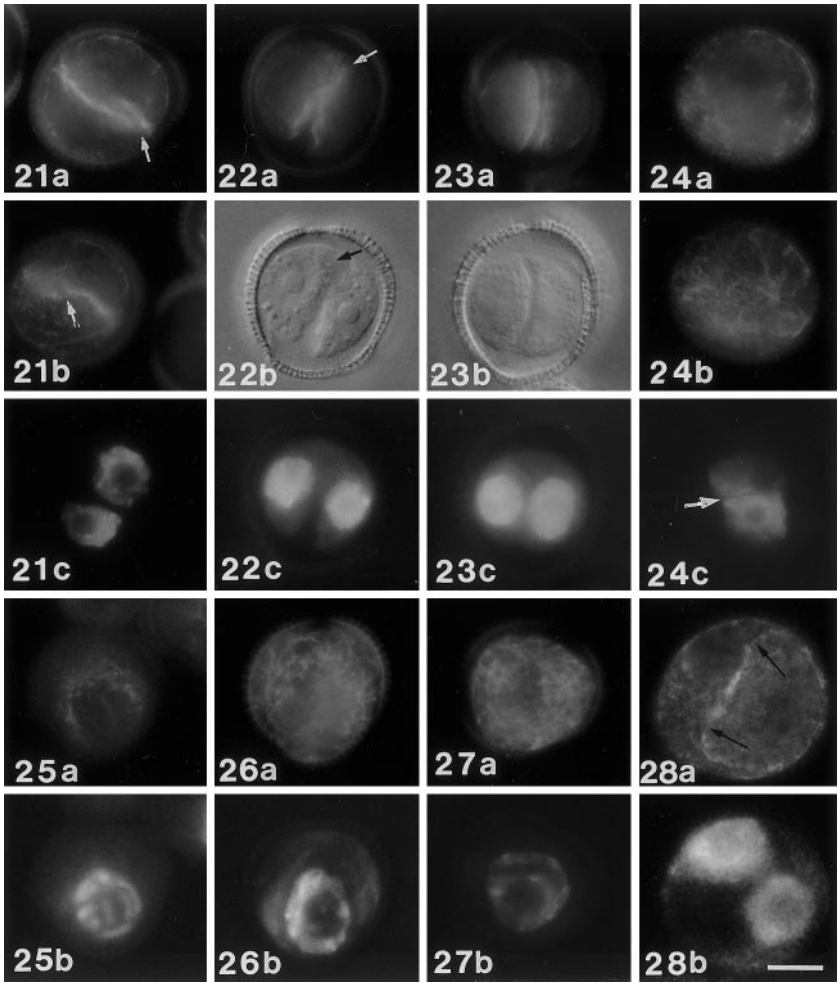
Fig. 18a,b. A microspore in telophase, after 16 h of culture, shows an equatorial MT phragmoplast (**a**) and similar chromatin condensation of the daughter nuclei (**b**)

Fig. 19a,b. A symmetrical bicellular structure after 24 h of culture shows very intense fluorescence at the cross-wall and cortical MTs at the surface of both cells (**a**). The nuclei show similar chromatin condensation (**b**)

Fig. 20a–c. The optical focal plane coincides with the plane of the cross-wall of a symmetrically divided structure and shows MT strands converging from the parental wall (**a**). Each nucleus is shown at its optical cross-section; the faint diffuse fluorescence (*arrows*) indicates the presence of the other nucleus (**b, c**)

trates this more clearly (Fig. 22b, arrow). The structure in Fig. 23 shows continuous cross-wall-associated fluorescence and a continuous cross-wall (Fig. 23a,b). Such continuous walls were found in 32–48% of the divided microspores (Table 1). At later culture times (48 h and later), some binucleate structures show no cross-wall (Fig. 24). The presence of binucleate structures without walls indicates that the wall had been resorbed because early divisions without walls were not observed.

Microtubule cytoskeleton in microspores of the non-embryogenic B. napus line 0025. Microtubule organization



Figs. 21–28. *Brassica napus* cv. Topas (Figs. 21–24) and the non-embryogenic line 0025 microspores (Figs. 25–28) cultured at 32.5 °C for 8–24 h except Fig. 24 which was cultured for 48 h. The cells were simultaneously labelled for MTs by immunofluorescence (Figs. 21a,b, 22a, 23a, 24a,b, 25a–28a) and for DNA with Hoechst 33258 (Figs. 21c–24c and 25b–28b), and viewed by DIC optics (Figs. 22b and 23b)

Fig. 21a–c. Binucleate structure with a discontinuous cross-wall. Discontinuous fluorescence at the new cross-wall shown at the optical cross-section (**a**, *arrow*), and at the cell surface (**b**, *arrow*), is indicative of a discontinuous wall. Similar condensation of daughter nuclei gives the illusion of a normal symmetric cell division (**c**)

Fig. 22a–c. Binucleate structure with an incomplete cross-wall. Cross-wall-associated fluorescence terminates before reaching the parental wall (*white arrow*; **a**). Cytoplasmic continuity between the two daughter 'cells' is more evident with DIC optics (*black arrow*; **b**). The similar appearance of the daughter nuclei gives the illusion of a normal symmetric cell division (**c**)

Fig. 23a–c. A bicellular structure with a continuous cross-wall. A symmetrically divided structure as shown by the continuous fluorescence at the cross-wall (**a**), the continuous cross-wall viewed by DIC optics (**b**) and similar condensation of daughter nuclei (**c**)

Fig. 24a–c. Binucleate structure with no cross-wall. A structure, after 48 h of culture, shows cortical, cytoplasmic and nuclear envelope associated MTs at the optical cross-section (**a**) and cortical MTs at the cell surface (**b**). The absence of a cross-wall is indicated by the absence of cross-wall-associated fluorescence (**a, b**) and by the close proximity of the two nuclei (**c**). A fine dark demarkation (*arrow*; **c**) separating the nuclei and the presence of two nucleoli confirm that karyokinesis had occurred

Fig. 25a,b. Microspore from the non-embryogenic line 0025, at prophase. No PPB is present and the perinuclear MTs are diminished and fragmented (**a**). The condensed chromosomes illustrate that the microspore is in prophase (**b**)

Fig. 26a,b. Late uninucleate microspore from the non-embryogenic line 0025. An optical cross-section shows fragmented MTs at the nuclear envelope (**a**). The absence of a vacuole (**a**) and the peripheral position of the nucleus (**b**) shows the developmental stage to be LU

Fig. 27a,b. A uninucleate microspore from the non-embryogenic line 0025. A reticulate network of cytoplasmic MTs at the optical cross-section (**a**) encompasses a centrally located nucleus (**b**)

Fig. 28a,b. A binucleate structure from the non-embryogenic line 0025. Discontinuous fluorescence (*arrows*) at the new cross-wall is indicative of a discontinuous wall (**a**). Similar condensation of daughter nuclei gives the erroneous appearance of a symmetric cell division (**b**)

of the non-embryogenic *B. napus* line 0025 was also examined during culture at 32.5 °C. No PPBs were found in these microspores at the LU and prophase stages (Fig. 25a). The appearance of a reticulate network of cytoplasmic MTs and a loss of perinuclear fluorescence was observed in LU microspores (Fig. 26) and in

microspores where the nucleus had moved to the centre of the cell (Fig. 27). Discontinuous, fragmented walls were present in cells which had undergone nuclear division (Fig. 28).

Discussion

Rearrangements of microtubules in heat-treated B. napus cv. Topas microspores. The data show that the MT organization of *B. napus* cv. Topas microspores developing as pollen is markedly different from that of microspores subjected to a heat treatment (32.5 °C, 24 h), which is required for induction of embryogenesis. Microspores developing in vivo and those cultured at 25 °C which resume pollen development, display perinuclear fluorescence and MT strands radiating from the surface of the nuclear envelope at all stages of microspore development through to the end of prophase. Fluorescence at the NC zone, denoting MT presence (Fig. 2a), increases dramatically during prophase (Fig. 4a). Microtubule involvement in anchoring the nucleus at the cell edge has been demonstrated using electron microscopy which showed MTs connecting the nuclear and plasma membranes (Hause et al. 1992) and by colchicine-induced MT depolymerization which resulted in nuclear displacement (Simmonds 1994). However, the maintenance of nuclear polarity is a complex process which undoubtedly requires MT interaction with other cytoskeletal components, e.g. microfilaments (Gervais et al. 1994).

Heat treatment of microspores changes MT organization. After 4–8 h of culture at 32.5 °C, perinuclear fluorescence is diminished and the nucleus is displaced from its peripheral position, indicating MT disruption at the NC zone; PPBs appear both in LU microspores and with prophase figures. Microtubules were disrupted in heat-treated non-embryogenic microspores of line 0025 and the nuclei were displaced; however, PPBs did not appear. Therefore, the PPB is a good indicator of embryogenic potential in microspores.

Role of PPBs in immobilizing cells. A PPB is formed prior to prophase in almost all tissues of higher plants, and its cortical location is the attachment site of the future cell plate (Picket-Heaps and Northcote 1966; Gunning 1982). By predicting division planes, PPBs have an important role in morphogenesis but they do not participate in the division of microspores (Van Lammeren et al. 1985; Terasaka and Niitsu 1990; Brown and Lemmon 1991a; Simmonds et al. 1991) and endosperm (De Mey et al. 1982). An additional role for PPBs, that of wall consolidation, has been proposed by Mineyuki and Gunning (1990). Using time-lapse microscopy, they demonstrated that the new wall in its formative stages is fluid and wrinkled, but minutes after it attaches to the parental wall, the wall becomes flat and rigid. Most significant to the present study, they showed that wall maturation does not occur if the cell plate is attached at a site not previously occupied by the PPB. They suggest that the PPB site becomes specialized by insertion of "factors" that promote wall maturation and consolidation. Stable wall formation is key in the development of multicellular organisms and underscores the significant difference between the two principal division mechanisms. Cell division by means of cell-plate development leads to stable walls and stationary cells within multicellular structures; whereas mobile single cells and unicellular organisms are the division products of cytokineses which do not form stable dividing walls (e.g. ingrowing cleavage furrows). Male gametogenesis is unique in higher plants because the cells generated are mobile. In some microspore cytokineses, the cell plate does not fuse with the parental wall (e.g. *Phalaenopsis*, Brown and Lemmon 1991b). In *B. napus*, the cell plate does fuse with the parental wall (Hause et al. 1992; Telmer et al. 1993); nevertheless, mobility of the generative cell is achieved. It is possible that this occurs because consolidated walls cannot form in the absence of PPBs and the accompanying "maturation factors". The unstable walls are diminished at later stages of development (Telmer et al. 1993), perhaps by the action of pollen specific wall-digesting enzymes expressed following mitosis (e.g. Robert et al. 1995). The appearance of the PPB in heat-treated microspores of the cv. Topas predicts a cytokinesis leading to a stable wall, a critical event in the initiation of a multicellular organism which is comprised of stationary cells separated by stable cell walls. Preprophase bands are not formed in heat-treated microspores of line 0025 and neither wall consolidation nor embryogenesis follow.

Significance of wall consolidation in division symmetry. Deviation from the normal asymmetric divisions, typical of the first pollen mitosis, accompanied induction of embryogenesis in many different plant species (Sangwan and Sangwan-Noreel 1987; Sunderland and Huang 1987; Fan et al. 1988; Zaki and Dickinson 1990, 1991; Telmer et al. 1993, 1995). These observation led to the suggestion that symmetric divisions blocked normal pollen development which resulted in a default developmental pathway leading to embryogenesis (Zaki and Dickinson 1990, 1991; Telmer et al. 1993, 1995; Simmonds 1994). However, the majority of microspores showing 'symmetric divisions' did not undergo embryogenesis; over 95% of microspore divisions were symmetric but only 7–16% were embryogenic (present report) and 10–30% were embryogenic in a previous study (Telmer et al. 1995). The present study has revealed that similar condensation of daughter nuclei does not necessarily signify symmetric cell divisions. Previous analyses of binucleate structures by electron microscopy or light microscopy of stained nuclei did not reveal information on the integrity of wall structures (Fan et al. 1988; Zaki and Dickinson 1990, 1991; Telmer et al. 1993, 1995; Touraev et al. 1995, 1996a, b, 1997; Zhao et al. 1996a). This report has provided evidence that more than half of the new cross-walls were abnormal as evaluated by DIC optics (Fig. 22b) and MT labelling at the cross-wall (Figs. 21a, 22a). Furthermore, some incomplete walls were resorbed at a later stage of development (Fig. 24). Since the method of

evaluation of wall integrity in this study was limited to only obvious and fairly large wall abnormalities, the score of 32–48% of microspores with continuous walls is most likely an overestimation as small flaws could not be seen. It is therefore conceivable that the percent of embryos reflects the true percent of continuous, consolidated walls. A structure with a discontinuous cross-wall is essentially a unicellular organism with two nuclei. The large number of structures without consolidated walls are binucleate cells, not "symmetrical divisions" because they are neither sporophytic nor gametophytic divisions. However, the structural and functional characteristics of these binucleate cells bear more resemblance to developing pollen grains as neither structure forms stable walls; also wall disintegration occurs in both structures (present study and Telmer et al. 1995), most likely mediated by pollen-specific genes (e.g. Robert et al. 1995). Likewise, the expression of pollen-specific promoters (Eady et al. 1995) and pollen tube growth (Touraev et al. 1995) reported to occur in "symmetrically divided" structures may have occurred in binucleate cells rather than in true symmetric divisions. It is therefore suggested that division symmetry where two daughter cells are separated by a consolidated, stable wall, is vital in the determination of a true bicellular structure.

Role of microtubule cytoskeleton in microspore embryogenesis. This study has shown that microtubule reorganization is a key event in the induction of embryogenesis. This conclusion supports a previous report which showed an increase in microspore embryogenesis by enhancing microtubule instability during the heat treatment by the inclusion of colchicine in culture media (Zaki and Dickinson 1991). Even more significant was the finding that embryogenesis can be induced with colchicine alone, without a heat treatment (Zhao et al. 1996a). Colchicine induction also disrupts spindles and phragmoplasts and genome doubling results; normal mitosis and cytokinesis resume after removal of colchicine (Zhao et al. 1996b).

It has been postulated that stress (Touraev et al. 1997), in this case heat stress, expressed by the production of heat-shock proteins (Cordewener et al. 1995 and references therein), is key to embryogenic induction. The validity of this hypothesis is questionable in view of the present study and the demonstration that colchicine can induce embryogenesis (Zhao et al. 1996a) without induction of heat-shock proteins (Zhao 1997). Stress may induce factors which alter the cytoskeleton, but the application of stress is not imperative because the cytoskeleton can be reorganized directly.

We have shown that, in *B. napus*, the PPB is both a marker of embryogenic potential and its integrity is critical to the development of the first consolidated wall which marks the beginning of a multicellular structure leading to embryogenesis.

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References

Baskin TI, Cande WZ (1990) The structure and function of the mitotic spindle in flowering plants. Annu Rev Plant Physiol Mol Biol 41: 277–315

Binarova P, Straatman K, Hause B, Hause G, van Lammeren AAM (1993) Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L. Theor Appl Genet 87: 9–16

Brown RC, Lemmon BE (1991a) Pollen development in orchids. 3. A novel generative pole microtubule system predicts unequal pollen mitosis. J Cell Sci 99: 273–281

Brown RC, Lemmon BE (1991b) Pollen development in orchids. 5 A generative cell domain involved in spatial control of the hemispherical cell plate. J Cell Sci 100: 559–565

Cordewener JHG, Hause G, Gorgen E, Busnik R, Hause B, Dons HJM, Van Lammeren AAM, Van Lookeren Campagne MM, Pechan P (1995) Changes in synthesis and localization of members of the 70-kDa class of heat-shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta 196: 747–755

De Mey J, Lambert AM, Bajer AS, Moeremans M, DeBrabander M (1982) Visualization of microtubules in interphase and mitotic plant cells of *Haemanthus* endosperm with the immunogold staining method. Proc Natl Acad Sci USA 79: 1898–1902

Eady C, Lindsey K, Twell D (1995) The significance of microspore divisions and division symmetry for vegetative-cell specific transcription and generative cell differentiation. Plant Cell 7: 65–74

Fan Z, Armstrong KC, Keller WA (1988) Development of microspores in vivo and in vitro in *Brassica napus* L. Protoplasma. 147: 191–199

Gervais C, Simmonds DH, Newcomb W (1994) Actin microfilament organization during pollen development of *Brassica napus* cv. Topas. Protoplasma 183: 67–76

Gunning BES (1982) The cytokinetic apparatus: its development and spatial regulation. In: Lloyd CW (ed) The cytoskeleton in plant growth and development. Academic Press, New York, pp 230–288

Hause G, Hause B, Van Lammeren AAM (1992) Microtubular and actin filament configurations during microspore and pollen development in *Brassica napus* cv. Topas. Can J Bot 70: 1369–1376

Hause G, Hause B, Pechan P, Van Lammeren AAM (1993) Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell Biol Int 17: 153–168

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Z Pflanzenphysiol 105: 427–434

Mineyuki Y, Gunning BES (1990) A role for the preprophase band of microtubules in the maturation of new cell walls, and a general proposal on the function of preprophase band sites in cell division in higher plants. J Cell Sci 97: 527–537

Picket-Heaps JD, Northcote DH (1966) Organization of microtubules and endoplasmic reticulum during mitosis and cytokinesis in wheat meristems. J Cell Sci 1: 109–120

Robert LS, Allard S, Gerster JL, Cass L, Simmonds J (1995) Isolation and characterization of a polygalacturonase gene highly expressed in *Brassica napus* pollen. Plant Mol Biol 23: 1273–1278

Sangwan RS, Sangwan-Noreel BS (1987) Ultrastructural cytology of plastids in pollen grains of certain androgenic and nonandrogenic plants. Protoplasma 138: 11–22

Simmonds DH (1994) Mechanism of induction of microspore embryogenesis in *Brassica napus*; significance of the preprophase band of microtubules in the first sporophytic division. In:

Akkas N (ed) Biomechanics of active movement and division of cells. NATO ASI series, vol H 84. Springer, Berlin, pp 569–574

Simmonds DH, Setterfield G (1986) Aberrant microtubule organization can result in genetic abnormalities in protoplast cultures of *Vicia hajastana* Grossh. Planta 167: 460–468

Simmonds DH, Gervais C, Keller WA (1991) Embryogenesis from microspores of embryogenic and non-embryogenic lines of *Brassica napus*. In: McGregor DI (ed) Rapeseed in a changing world. Proceedings of the Eigth International GCIRC Rapeseed Congress, Saskatoon, Sask. Canada, July 9–11, 1991. Groupe Consultatif International de Recherche sur la Colza (GCIRC) and the Canola Council of Canada, pp 306–311

Sunderland N, Huang B (1987) Ultrastructural aspects of pollen dimorphism. Int Rev Cytol 107: 175–220

Telmer CA, Simmonds DH, Newcomb W (1992). Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. Physiol Plant 84: 417–424

Telmer CA, Newcomb W, Simmonds DH (1993) Microspore development in *Brassica napus* and the effect of high temperature on division *in vivo* and *in vitro*. Protoplasma 172: 154–165

Telmer CA, Newcomb W, Simmonds DH (1995) Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. Protoplasma 185: 106–112

Terasaka O, Niitsu T (1990) Unequal cell divisions and chromatin differentiation in pollen grain cells II. Microtubule dynamics associated with the unequal cell division. Bot Mag Tokyo 103: 133–142

Touraev A, Pfosser M, Vicente O and Heberle-Bors E (1995) Maintenance of gametophytic development after symmetrical division in tobacco microspore culture. Sex Plant Reprod 8: 70–76

Touraev A, Pfosser M, Vicente O, Heberle-Bors E (1996a) Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis. Planta 200: 144–152

Touraev A, Indrianto A, Wratschko I, Vicente O, and Heberle-Bors E (1996b) Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) Induced by starvation at high temperatures. Sex Plant Reprod 9: 209–215

Touraev A, Vicente O Heberle-Bors E (1997) Initiation of microspore embryogenesis by stress. Trends Plant Sci 8: 297–302

Van Lammeren AAM, Keijzer CJ, Willemse MTM, Kieft H (1985) Structure and function of the microtubular cytoskeleton during pollen development in *Gasteria verrucosa* (Mill.) H. Duval. Planta 165: 1–11

Zaki MAM, Dickinson HG (1990) Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma 156: 149–162

Zaki MAM, Dickinson HG (1991) Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex Plant Reprod 4: 48–555

Zhao J-P (1997) Induction and mechanism of *Brassica napus* cv. Topas microspore embryogenesis. Ph.D. Thesis, Queen's University, Kingston, Ont, Canada

Zhao J-P, Simmonds DS, Newcomb W (1996a) Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L. cv. Topas. Planta 198: 433–439

Zhao J-P, Simmonds DS, Newcomb W (1996b) High frequency production of doubled-haploid plants of *Brassica napus* cv. Topas derived from colchicine-induced microspore embryogenesis without heat shock. Plant Cell Rep 15: 668–671