Protoplasma
DOI 10.1007/s00709-008-0007-6

ORIGINAL ARTICLE

Recovery of microtubules on the blepharoplast of *Ceratopteris* spermatogenous cells after oryzalin treatment

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Received: 20 February 2008 / Accepted: 13 June 2008

Abstract Most land plants have ill-defined microtubule-organizing centers (MTOCs), consisting of sites on the nuclear envelope or even along microtubules (MTs). In contrast, the spermatogenous cells of the pteridophyte *Ceratopteris richardii* have a well-defined MTOC, the blepharoplast, which organizes MTs through the last two division cycles. This allows a rare opportunity to study the organization and workings of a structurally well-defined plant MTOC. In this study, antheridial plants were treated with levels of oryzalin that cause complete MT loss from the cells containing blepharoplasts. The oryzalin was then washed out and plants were allowed to recover for varying amounts of time. If the spermatogenous cells were fixed prior to washing out, the blepharoplasts had an unusual appearance. In the matrix (pericentriolar) material where MT ends are normally found, clear areas of about the diameter of MTs were seen embedded in a much deeper matrix, made more obvious in stereo pairs. Occasionally, the matrix material was highly distended, although the basal body template cylinder morphology appeared to be unaltered. The blepharoplasts often occurred as clusters of 2 or 4, indicating that blepharoplast reproduction is not affected by the lack of MTs, but that their movement to the poles is. Gamma (γ) tubulin antibodies labeled the edge of the blepharoplast in areas where the pits are located, indicating that these might be sites for MT nucleation. After wash out, the new MTs always re-appeared on the blepharoplast and the recovery occurred within an hour of washout. MT lengths increased with increasing washout time and were indistinguishable from untreated blepharoplasts after 24 h of recovery. After washout, arrays formed in new sperm cells such as the spline and basal bodies were often malformed or present in multiple copies, as were the blepharoplasts in these cells prior to wash out. These data indicate that the blepharoplast serves as the site of MT nucleation and organization even after complete MT de-polymerization.

Keywords Microtubule organizing center · Spermatogenous cells · Pteridophyte · Oryzalin

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Introduction

Plant microtubule-organizing centers (MTOCs) are one of the most difficult areas of plant cell biology because, unlike mammalian cells where a centrosome is clearly present, no homologous structure is detectable at the spindle poles or in any other array in most higher plants (Vaughn and Harper 1998). This has lead to a variety of theories as to the nature of plant MTOCs, most relying on either the nuclear envelope or other endomembranes for sites of microtubule (MT) nucleation (Lambert 1993; Vaughn and Harper 1998; Erhardt et al. 2002; Schmit 2002), although the subsequent final site (or docking) might be on another structure. An example would be the generation of MTs on the surface of the nuclear envelope but the final site of deposition being along the cortical cytoplasm/plasma membrane interface (Lambert 1993; Vaughn and Harper 1998). More recently, a new theory (Murata et al. 2005, 2007) has been developed based upon the observation that γ tubulin appears to be found along MTs, rather than just at their site of origin (Liu et al. 1993; Hoffman et al. 1994). This theory assumes that γ tubulin forms a complex at points along the MT, allowing MTs to branch and essentially nucleate new MTs from

existing ones. In fact, the localization of γ tubulin published by Vaughn and Harper (1998; Fig. 15) and earlier by Hoffman et al. (1994) was consistent with this hypothesis, although the authors did not recognize its significance at that time. These data also explain some of the "fir tree patterns" of MTs found in higher plants, in which MTs appear to have converged or to have formed at an angle from or near existing MTs (Palevitz 1993).

Although higher land plants do not have a recognizable MTOC, lower land plants have easily recognizable structures present at the minus end of MTs, such as blepharoplasts, polar organizers, and amorphous areas of pericentriolar material (Vaughn and Harper 1998; Brown and Lemmon 2007). The localization of γ tubulin, centrin, and other centrosomal proteins at these sites (Vaughn et al. 1993; Hoffman et al. 1994; Klink and Wolniak 2003; Shimamura et al. 2004) confirms their identity as MTOCs. RNAi work has confirmed the theory that the presence of at least one of these proteins is critical for MT organization in these plants (Klink and Wolniak 2001). As these structures are well-defined and widely accepted by other scientists as MTOCs, they allow us to answer some basic questions that are not easily addressed by studying plants that have less well-defined MTOCs.

In the pteridophytes, the structure known as the blepharoplast arises in the next to last mitotic event in spermatogenous cell development (Hepler 1976; Hoffman and Vaughn 1996). It organizes the interphase array of MTs and serves as the spindle organizer for the two final mitotic divisions. Prior to each of these divisions, the blepharoplast divides and moves to the poles, serving as the focus for an astral spindle. The blepharoplast is spherical, approximately 0.5 μm in diameter, with barrel-like structures that serve as centriolar templates embedded in an electron-opaque pericentriolar matrix. After the final spermatogenous cell division, the blepharoplast re-organizes into a much looser structure and basal bodies are produced on the basal body templates. From these basal bodies, the sperm flagellar apparatus is organized around the two new MTOCs that are derived from the blepharoplast, the multi-layered structure and the spline. Thus, the blepharoplast enables both the organization of the interphase and mitotic array of the spermatogenous cells, and in its reorganized form, the production and organization of the flagellar apparatus.

In this study, we examined *Ceratopteris* spermatogenous cells that had been treated with the MT disrupter oryzalin and then allowed to recover for varying periods. Antheridial plants form spermatogenous cells in synchrony, so that, within a group of such cells, all are at the same developmental stage, greatly facilitating the analysis of both the oryzalin disruption and subsequent recovery. These samples were examined by traditional electron microscopy, immunocytochemistry, and by stereo microscopy of tilted specimens.

Materials and methods

Plant material and treatment Spores of *Ceratopteris richardii* were sown on agar medium covered with a thin membrane, as described previously (Hoffman and Vaughn 1996). After 7–10 days of growth, the membrane and attached plantlets were removed from the agar surface and either placed in 0.1% acetone (solvent control) or 10 μM oryzalin (prepared from 10 mM stocks in acetone) for 24 h in a new Petri dish with a piece of moistened Whatman's #1 filter paper. (This concentration and time were chosen using the results of a set of preliminary experiments in which MT disruption was monitored. These conditions resulted in the complete loss of MTs with the exception of those in acetylated arrays in the sperm cells.) The samples were then either prepared for analysis or transferred into a new Petri dish with distilled water only for various times from 2–24 h to allow for recovery. These experiments were repeated three times and a minimum of a dozen antheridial plants were sampled from each experiment.

Microscopy Samples were fixed by flooding the cultures with 6% (v/v) glutaraldehyde in PIPES buffer (pH 7.4) for 2 h. The antheridial plantlets were transferred to scintillation vials and then rinsed with 0.10 M cacodylate buffer (pH 7.2), twice for 15 min each. The samples were post fixed in 2% (w/v) OsO_4 in cacodylate buffer for 2 h. After a water rinse, the samples were dehydrated in acetone and transferred to propylene oxide before being embedded in a 1:1 mixture of Spurr's low viscosity resin and Polybed 812 (Polysciences Inc. Warrington PA). Samples were infiltrated with 25, 50 and 75% plastic for 2 h each and then covers of aluminum foil with small holes in the surface were placed over the sample vials so that the propylene oxide could volatilize, gradually increasing the proportion of plastic for an additional 18 h. Samples were then transferred into fresh 100% resin and shaken on a rocking platform for 24 h before polymerization in fresh resin in BEEM capsules.

Blocks were sectioned with a Reichert Ultracut ultramicrotome using Delaware diamond knives. Light microscopy of semi-thin sections (0.35 μm) stained with 1% toluidine blue (in 1% sodium borate) was used to determine the presence of antheridial plants at the proper stage of development. Thin (~100 nm) sections were mounted on uncoated copper grids or Formvar-coated slot grids (for serial sections) and examined with a Zeiss EM10CR electron microscope operating at 60 kV.

To obtain stereo images through the blepharoplast, the grids were positioned in the goniometer cartridge of the Zeiss microscope and an axis that ran diagonally through the blepharoplast was used as a fulcrum for titling the sample. Blepharoplasts were photographed at tilt angles from −40° to +8°. A pair of images 5° apart that showed

optimal detail of the blepharoplast after oryzalin treatment was chosen. These negatives were scanned and used to construct anaglyphs using the GIMP.

For quantification of MT lengths, a minimum of 12 micrographs of blepharoplasts from each time point of recovery (0–24 h) was taken. Data are expressed as the mean length of the longest MTs on each blepharoplast.

Immunocytochemistry Samples were prepared as described by Hoffman et al. (1994) utilizing the 0.6% glutaraldehyde fixation that results in superior immunolabeling with the γ tubulin antiserum. Samples were fixed in 0.6% glutaraldehyde in 0.05 M PIPES buffer (pH 7.2) for 1–2 h at room temperature, washed in PIPES buffer and dehydrated in an ethanol series at 4°C. After 100% ethanol had been reached, the samples were transferred to a −20°C freezer and LR White resin was added in 25% increments, for 24 h each, with a final day at 100% resin. The samples were transferred to a rocking platform at room temperature for 24 h to enhance resin infiltration. They were then transferred to BEEM capsules and polymerization took place at 55°C in a vacuum oven. After 2 h, polymerization was complete. Thin sections were mounted on uncoated 300 mesh gold grids and processed as described previously (Hoffman et al. 1994). Briefly: 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), 30 min; primary antibody (γ tubulin [1:80], centrin [1:80], MPM-2 [1: 10], a mixture of tubulin monoclonals [1: 40]) diluted in PBS-BSA, 3 h; 4 washes in PBS-BSA, 2.5 min each; Protein A gold, or goat-anti rat or mouse IgG coupled to 125 nm colloidal gold, 1 h. All buffer solutions were filtered with a 0.2 μm filter prior to the experiment. The sections were then floated on drops of double-distilled water and then washed extensively with double-distilled water from a squirt bottle. The grids were dried on Whatman's #1 filter paper and post-stained with uranyl acetate (2 min) and Reynolds's lead citrate (30 sec) before observation with a Zeiss EM 10 CR electron microscope operating at 60 kV. Data from 23 micrographs were collected from the grid holes nearest the 500/500 centering point on the microscope to determine the density of the immunogold labeling around the blepharoplast. A normal rabbit antiserum, diluted as the primary serum, served as the control for the γ tubulin and centrin antisera, whereas an irrelevant serum or no primary

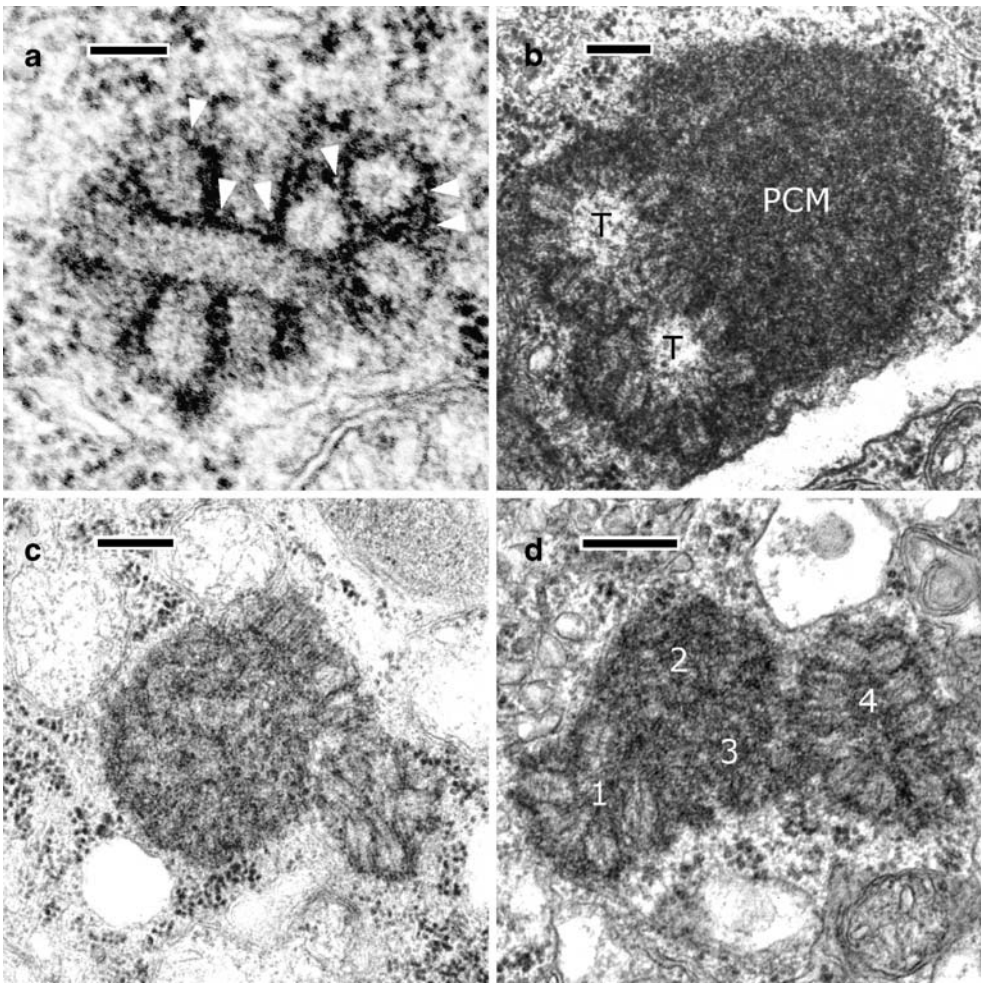

Fig. 1 Examples of variable morphology after oryzalin treatment. **a** Blepharoplast with very pitted pericentriolar material and cylinders that are basal body templates. Putative nucleating sites are indicated by *white arrowheads*. Scale bar=0.1 μm. **b** Divided blepharoplast with pericentriolar material (PCM) and distinctly separated basal body templates (T). Scale bar=0.25 μm. **c** Enlarged (possibly arrested in division) blepharoplast with good basal body template morphology. Scale bar=0.25 μm. **d** A multi-blepharoplast complex (blepharoplasts labeled *1–4*) that resulted from sequential division but no separation. (This section was used to make the anaglyph shown in Fig. 2.) Scale bar=0.1 μm

serum served as the control for the monoclonal antibodies. None of these produced labeling over 0.3 gold particles/μm^2 over the cell walls, which were the greatest areas of background labeling.

Results

Blepharoplast structure after oryzalin treatment

Ceratopteris antheridia treated with oryzalin for 24 h were devoid of MTs with the exception of the highly stabilized arrays of the spline and flagellar MTs. The resistance of these structures to MT disrupters has been described in a previous report (Hoffman and Vaughn 1996). This report is concerned primarily with the MTOC known as the blepharoplast, the effects of treatment with oryzalin, and their subsequent recovery. In contrast to the oryzalin-resistant MTs seen in other spermatogenous cell-specific MT arrays, MTs emanating from the blepharoplast are lost after oryzalin treatment (Fig. 1), despite the presence of a functioning MTOC, which should theoretically block depolymerization at the minus end. The MT-less blepharoplast can resemble a blepharoplast that is otherwise unaffected (Fig. 1a) but may also be slightly stretched (Fig. 1b). If blepharoplast division has occurred with no subsequent migration to the poles, it may occur as a complex of multiple blepharoplasts (Fig. 1c,d). Up to four blepharoplasts were observed in a given clump in a single section or from sets of serial sections, indicating that at least two division cycles can occur without movement of this MTOC.

These multi-blepharoplast complexes could be distinguished from the re-organized blepharoplasts (Hoffman and Vaughn 1996) that occur in the transition from the final mitotic division by several criteria: (1) the multi-blepharoplast complex contains obvious spherical blepharoplast structures, (2) there is no central core of pericentriolar material and ring of basal body templates, as in true re-organizing blepharoplasts, and (3) the multi-blepharoplast complex does not contain centrin, whereas re-organizing blepharoplasts do (Hoffman et al. 1994). Moreover, during our study of untreated *Ceratopteris* spermatogenous cells, antheridia that were in the re-organizing blepharoplast stage were relatively rare. In fact, this stage is only recognizable by the presence of centrin within the blepharoplasts. In contrast, multi-blepharoplast complexes were observed in nearly 30% of the treated antheridial cells. Within a given antheridium, all of the spermatogenous cells displayed very similar blepharoplast morphology, which is consistent with the synchrony that is observed within these cells when not grown in the presence of oryzalin (Hoffman and Vaughn 1995b). That is, when four blepharoplasts were observed in one spermatogenous cell, four were also observed in all of the others in a given antheridium. Because four is the number of blepharoplasts generated by two rounds of unsuccessful division, and we observed no more than four blepharoplasts in a given cell, the data fit most tightly with the theory that the blepharoplasts are able to reproduce without MTs but their movement is restricted.

One of the most unusual aspects of the MT-less blepharoplast was the appearance of electron-translucent pits (Fig. 1a) in a distribution pattern similar to that of the MTs themselves on an untreated blepharoplast. These pits were also ~25 nm in diameter, which is the approximate diameter of the MTs. In the oryzalin-treated blepharoplasts that appeared relatively similar in structure to untreated controls (with the exception of attached MTs), the pits were regularly distributed over the blepharoplast surface. In other cells, however, the blepharoplast surface had been stretched and/or multiple blepharoplasts were present. The distribution of the pits was also affected, sometimes occurring as clusters on the edge of the distended blepharoplast (Fig. 1b,d). The pericentriolar matrix material was also displaced, sometimes stretched out to such an extent that holes filled with cytoplasm were found within the blepharoplast or its components were very segregated, with the matrix present at one end and the basal body templates at another (Fig. 1b). The cylinders that serve as the template for the basal body were mostly observed as intact structures, although their distribution in the distended blepharoplasts was often also abnormal.

In order to observe a more three-dimensional view of the blepharoplast after oryzalin treatment, stereo pairs were used to create anaglyphs using a plane through the four blepharoplasts as the fulcrum for the tilt series. A section at higher magnification from that used in the tilt series is shown for comparison (Fig. 1d). In the anaglyph images, the basal body template cylinders may be observed as lying in the electron-opaque pericentriolar matrix (Fig. 2).

At stages of spermatogenous cell development prior to the appearance of a blepharoplast, the cells respond to oryzalin in exactly the same way as other land plant cells lacking detectable MTOCs (Hoffman and Vaughn 1996). Loss of all MTs occurs, causing arrested prometaphase cells and production of lobed nuclei and aberrant phragmoplasts. These results, and the lack of effects on mature sperm cells, are consistent with our previous studies of mitotic disrupter-treated *Ceratopteris* cultures (Hoffman and Vaughn 1995b).

Recovery of MTs on blepharoplasts

If the blepharoplast does represent an MTOC, then MTs should rapidly appear on its surface after the removal of the MT polymerization inhibitor oryzalin (Fig. 3). For these studies, only cells with single blepharoplasts were used. Cells with a multi-blepharoplast complex converted directly

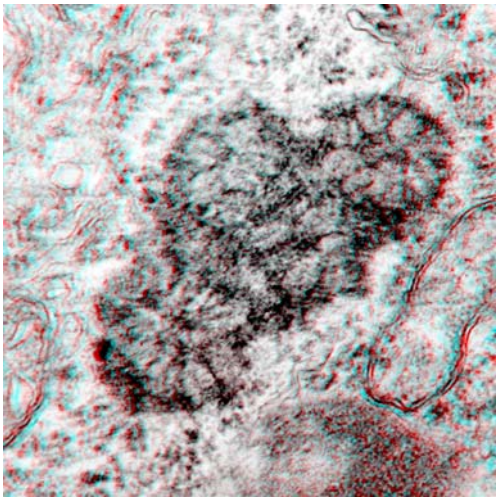

Fig. 2 Anaglyph prepared from tilted samples of the section shown in Fig. 1d. Note that the basal body templates appear to sit in the much more electron-opaque pericentriolar matrix. The pits appear as very shallow structures in the matrix and only towards the periphery

to a re-organizing blepharoplast and began organizing basal bodies (see below). We monitored cultures at 2, 4, 6, 12 and 24 h after washing out the oryzalin solution. Even after 2 h recovery from oryzalin, MTs as long as 1.7 μm were noted (Table 1) emerging from the blepharoplast, although most were much shorter. Because the MT lengths were calculated from electron microscope sections, we were only able to measure the length of MTs that happened to run in the plane of the section. Some MTs may actually traverse more than one section. Thus, the lengths reported in Table 1 may be somewhat shorter than the actual lengths of the recovering MTs. Even with this caveat, the recovery of the MTs was very rapid, with essentially untreated control lengths observed after only 24 h (Table 1). However, some of the blepharoplasts observed after 24 h of recovery could have arisen de novo during the recovery period, which might skew these numbers more towards the untreated levels. Despite what must be a relatively high concentration of free tubulin directly after treatment, no MTs were formed in areas of the cell away from the blepharoplast (also confirmed independently by immunofluorescence microscopy of methacrylate sections, unpublished). Thus, the blepharoplast serves as a site for MT recovery even though in previous cell generations the same tubulin subunits would have organized MTs at other cellular sites.

As mentioned above, the blepharoplasts often duplicate during the oryzalin treatment and then remain as a clump of duplicated structures. Upon recovery, these duplicated blepharoplasts go through the normal transition into a multiple re-organized blepharoplast (Hoffman and Vaughn 1995a), and subsequently produce motile apparatuses for each of the re-organized blepharoplasts present in a cell (Fig. 4). For example, some of the recovered samples at 12 and 24 h after oryzalin wash out had multiple multi-layered structures complete with multiple growing splines and associated flagella (Fig. 4c,d), each oriented in a different direction or location within the cell (Fig. 4a,b). Thus, these data indicate that once the MTOC material has duplicated, this also causes a multiplicity in subsequent MTOCs, even though their composition and structure are different (e.g. centrin is found in the stages after blepharoplast formation but not before). Sometimes the MLSs formed twinned structures (Fig. 4c), whereas in other cells they were on different sides of the cell (Fig. 4a) or nearby but perpendicular to each other (Fig. 4d). Although some of this distribution may reflect the relationship with a larger, more lobed nucleus, one of the flagellar apparatuses in Fig. 4b is associated with a nucleus and the other is not. We did not follow recovery past 24 h, so the fate of these cells

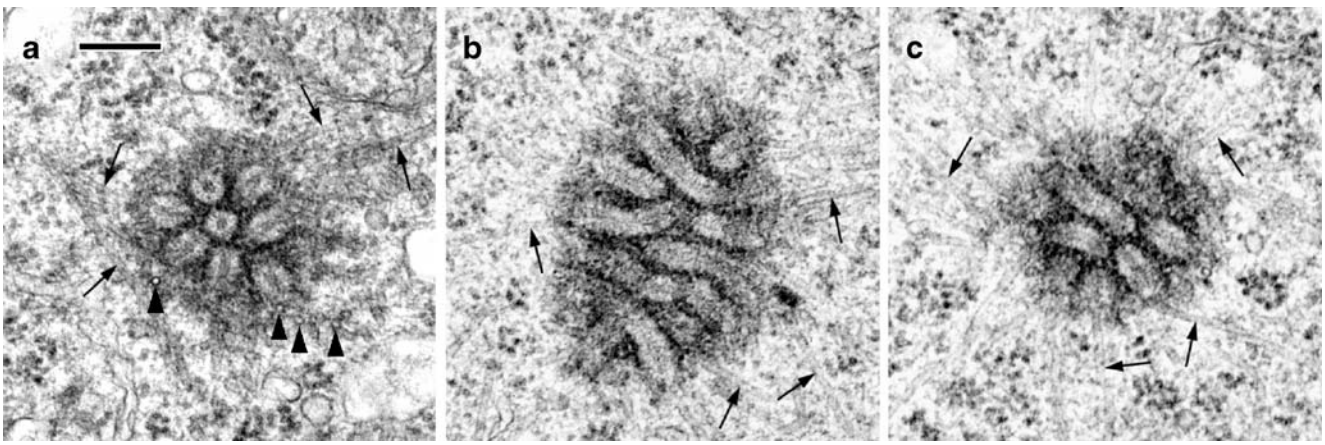

Fig. 3 Blepharoplasts recovering from oryzalin treatment 2 (**a**), 6 (**b**) and 12 h (**c**) after washout. Small MTs, and later larger MTs, are seen emanating from the surface of the blepharoplast (*arrows*). *Arrowheads* point to MTs running perpendicular to the plane of the section. Scale bar=0.2 μm

Table 1 Lengths of MTs that surround the interphase blepharoplast after recovery from oryzalin treatment

Time of recovery (h)	MT length
0	0±0
2	1.06±0.4
4	2.2±0.45
6	4.7±0.47
12	6.2±0.3
24	6.4±0.4
Control	6.6±0.6

MT lengths were measured on TEM sections from at least 12 different interphase blepharoplasts from each of three experiments, and the longest MTs were measured. Controls were from a non-oryzalin-treated sample prepared on the same day and examined in the same manner. Data is expressed as average MT length in μm±SE

with highly disorganized flagella was not studied further. However, the many examples of deviant basal body formation in the specimens 12–24 h after the removal of the oryzalin (Fig. 5b) indicate strongly that subsequent flagellar development would also be affected. Certainly, the presence of multiple spline and MLS complexes found in the recovered cells should result in sperm with too many flagella and/or flagella organized in an abnormal manner.

In stages prior to the appearance of a blepharoplast, the cells recover much in the same way as other land plant cells (e.g. Cleary and Hardham 1988). MTs re-appear, sometimes on the nuclei but apparently at cortical sites as well, or at least so fast that the production and movement occurs in less than 4 h from the initial washout of oryzalin.

Immunocytochemistry of the oryzalin-treated blepharoplast and subsequent arrays

Blepharoplasts denuded of MTs by oryzalin were probed with antibodies/antisera that recognize MTOCs. Reaction was noted to several when a very low concentration of glutaraldehyde was used for fixation. Most interesting is the labeling of γ tubulin on the blepharoplast (Fig. 6a,b). Here the label was confined to the surface of the blepharoplast, although in those blepharoplasts where the structure had expanded during the oryzalin treatment, the label appeared to be in patches rather than spread evenly around the periphery, like the ~25 nm pits. Thus, γ tubulin retains its association with the MTOC despite the disruption of the MTs with oryzalin and further loss of integrity during attempts at division. In contrast, the centrosomal phosphoprotein antibody MPM-2 labeled the electron-opaque pericentriolar material but not the edge of the MT-denuded blepharoplast (Fig. 6c). However, this MTOC antibody also binds to the structure despite the loss of MTs. In our previous studies (Hoffman et al. 1994), due to the tight association of the basal body cylinders and the pericen-

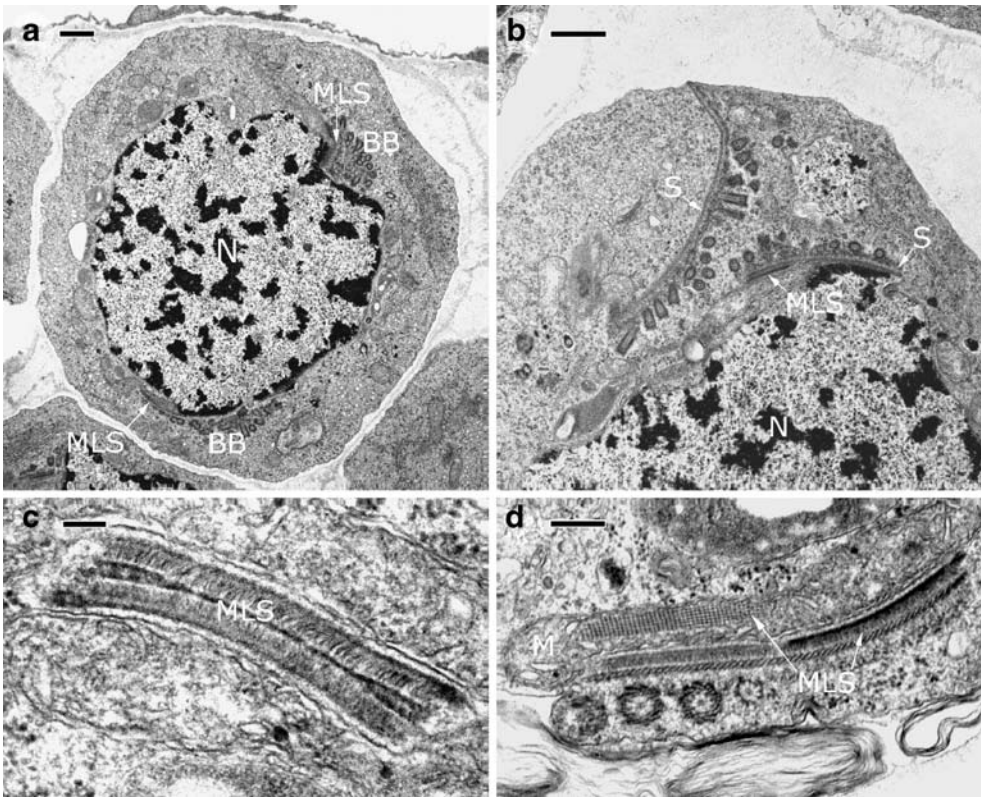

Fig. 4 Examples of recovery from oryzalin treatment in the sperm cell stage, 12 (**a**, **c**) or 24 h (**b**, **d**) after washout. **a** Instead of a single flagellar apparatus, a clump of flagellae, spline MTs, and a multilayered structure is found at each pole. Scale bar=1 μm. **b** Side-by-side flagellar apparatuses, one associated with the nuclear envelope, one not. Scale bar=1 μm. **c** Duplicated multilayered structures, each associated with a mitochondrion. Scale bar=0.1 μm. **d** Duplicated MLSs each associated with a mitochondrion (*M*) but at right angles rather than twinned as in Fig. 4c. Scale bar=0.5 μm. *MLS* Multilayered structure, *BB* basal bodies, *N* nucleus, *S* spline

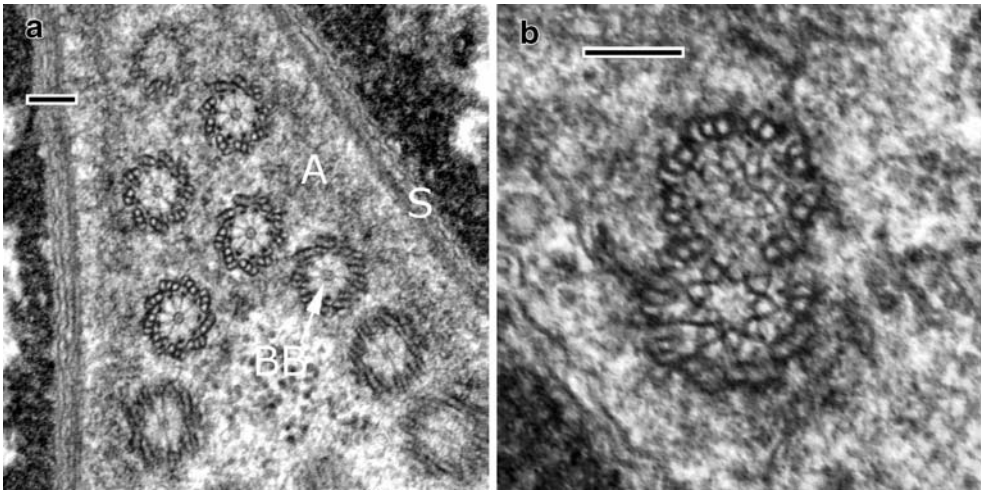

Fig. 5 Oryzalin washout and its effects on basal body distribution and morphology. **a** Two separate flagellar bands can be seen adjacent to the two splines (*S*), although basal body (*BB*) morphology appears normal. *A* amorphous zone. Scale bar= 0.1 μm. **b** Doubled basal body in the stellate pattern zone, in which the doublet MTs are shared between two adjacent flagella. Scale bar=0.1 μm

triolar material in the blepharoplast, it was impossible to determine exactly where MPM-2-reactive phosphoproteins were localized. However, in this study, where the blepharoplast components were spread out, the basal body templates were clearly unlabeled even at a relatively high antibody concentration. Antibodies and antiserum to other tubulins reacted neither with the blepharoplast interior nor with its surface (Pennell et al. 1986; Hoffman et al. 1994, and data not shown).

In previous studies, we have shown that centrin appears in the re-organizing blepharoplast just after the final spermatogenous cell division (Hoffman et al. 1994), in the MLS, and in the amorphous zone in the sperm cell (Vaughn et al. 1993). In the recovered sperm cells, centrin was found on the now multiple re-organizing blepharoplasts, MLS, and amorphous zones (Fig. 6d). Thus, despite the abnormal appearance and distribution of these structures, their composition, at least with respect to the centrin component, is similar to those in untreated cells.

Discussion

Structure of the MT-less blepharoplast

Treating the antheridial plants of *Ceratopteris richardii* with oryzalin results in the removal of all MTs, from the stages of development up to and including the appearance of the blepharoplast at the penultimate division stage. The absence of MTs from the surface of the blepharoplast is a little surprising, as one would expect that this structure would cap the minus end of the MTs and prevent complete depolymerization. Blepharoplast structure is affected in several ways by oryzalin treatment. On the surface of the blepharoplast, in the spaces occupied by MTs in the untreated controls, only electron translucent pits of about 25 nm were found studding the surface (Fig. 1a). In other cases, the pits occurred in a less regular pattern because the blepharoplast or the pericentriolar material had stretched or become displaced during the oryzalin treatment (Figs. 1b and 2). Blepharoplasts contain no α or β tubulin (Pennell et al. 1986; Hoffman et al. 1994; and unpublished results) and γ tubulin is only found at the periphery and along MTs in untreated cells (Hoffman et al. 1994). Thus, the pits are not themselves tiny MTs.

In addition, blepharoplasts seem to reproduce themselves as they might have done in a cell responding to the normal cell signals, but their movement to the poles is prevented due to a lack of MTs. Up to four blepharoplasts were noted in a single section (Figs. 1d and 2), indicating that at least two potential division events were thwarted by the oryzalin treatment, and that two cycles of blepharoplast replication had occurred. In some cases, the underlying pericentriolar material had become stretched, leaving the basal body templates in one area (Fig. 1b), but in others, as in the example in the anaglyph (Fig. 2), even the four blepharoplasts had a relatively normal morphology despite two rounds of replication.

Duplication of the MTOC and its effect on recovery

One of the characteristics of MTOCs is their ability to duplicate at a cell-specific time during development, presaging the impending mitotic division (Bornens 1992). In most plant cells, there is no reliable marker for this duplication of centrosomal material, but such a marker exists in the case of the blepharoplast. In normal division cycles, the blepharoplast divides twice in the last two spermatogenous cell divisions (Hepler 1976; Hoffman and Vaughn 1995b). Each time, the blepharoplasts move to the poles and organize the spindle apparatus for the ensuing division. The movement of the blepharoplast to the poles, but not their duplication, is MT-dependent.

Oryzalin treatment severely affects the ability of the blepharoplasts to move after replication. As many as four blepharoplasts may be identified in a given section (or set of serial sections), indicating that the duplication that would have occurred in the last two cell divisions occurs despite the lack of MTs. Most interestingly, this multiple blepharoplast complex (Fig. 1d) also results in the multiplicity of subsequent MTOCs in the mid-stage spermatogenous cell (Fig. 4). In this case, the presence of a multi-blepharoplast complex leads to multiple re-organizing blepharoplasts and, subsequently, to multiple flagellar apparatuses. This change of one MTOC to another may be what is set in motion by cell cycle events regardless of the number of these MTOCs, i.e. the cellular signals for conversion had occurred despite the duplication (but not separation) of MTOC material. This mechanism may also ensure the correct amount of MTOC material in cells going through endopolyploidy during normal somatic cell development.

Comparison with other species

MT disruption and recovery has been previously investigated in systems with well-defined MTOCs, especially in the bryophytes (Busby and Gunning 1989; Shimamura et al. 2004). In the spore mother cells of the moss *Funaria*, the MTs emanate from the tips of plastids or perhaps from lipidic substances that concentrate at the tips of the plastid envelope (Busby and Gunning 1989). Treatment with oryzalin results in the loss of the MTs from this MTOC. Recovery from oryzalin treatment was nearly always

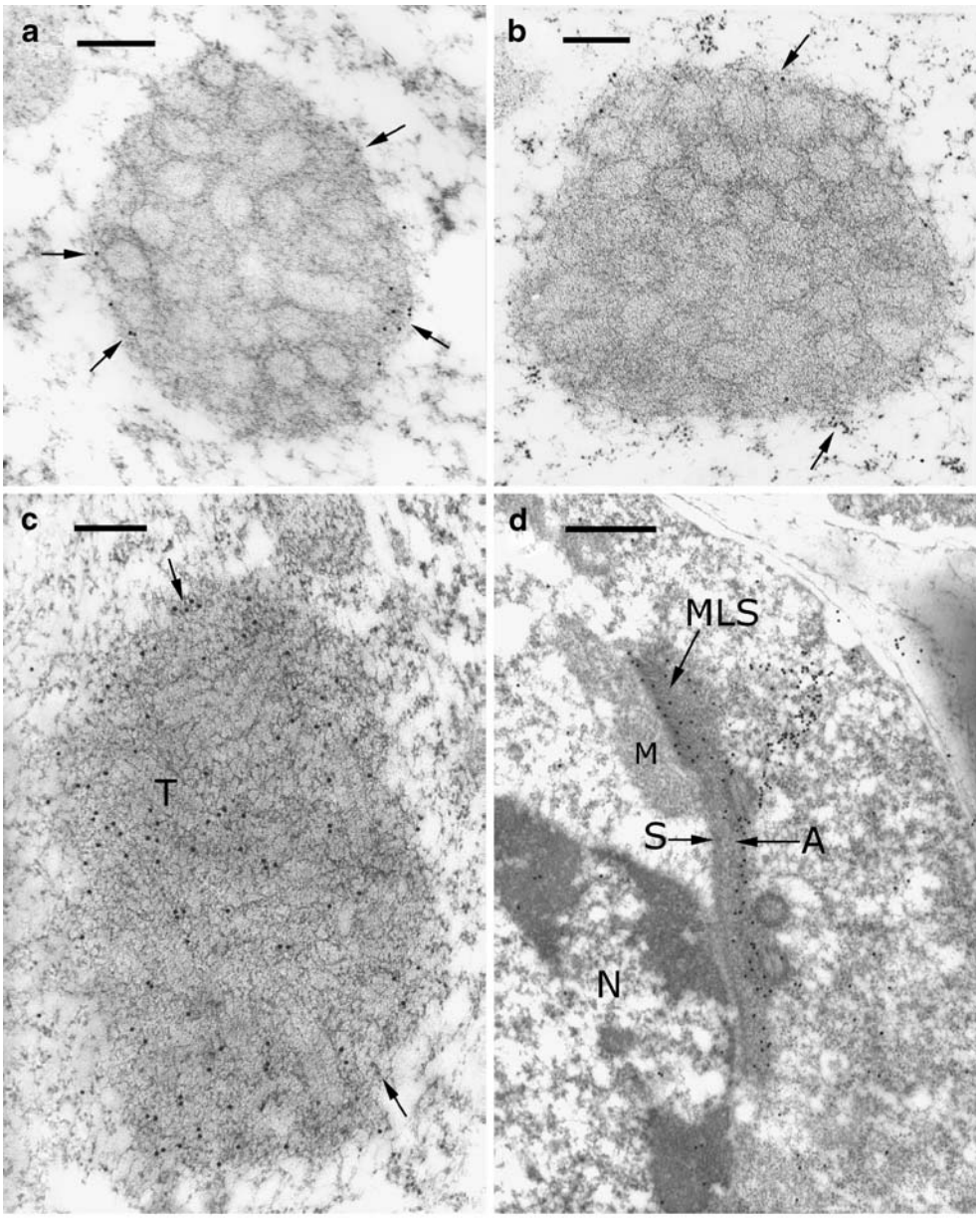

Fig. 6 Immunocytochemistry of oryzalin-treated spermatogenous cells (**a**–**c**) and after 12 h recovery (**d**). **a** and **b** γ tubulin localization on MT-less blepharoplasts. Scale bar=0.5 μm for a and 0.25 μm for **b**. Antibody binding only occurs in the pericentriolar material near the blepharoplast periphery and not on the basal body template cylinders. **c** In contrast, MPM-2, which recognizes a pericentriolar matrix phosphoprotein, binds throughout the pericentriolar matrix but does not label the basal body templates. *T* basal body template. Scale bar=0.25 μm. **d** In a recovered sperm cell, centrin is associated with the spline (*S*) and the multilayered structure (*MLS*) but also in patches away from these structures. *M* Mitochondrion, *N* nucleus, *A* amorphous zone. Scale bar=0.5 μm

associated with the first re-appearance of MTs occurring at these sites, an exception being re-growth of captured MTs at kinetochores (Busby and Gunning 1989). Interestingly, division of the plastids (and the MTOC) was not affected by oryzalin, although the shapes and disposition of the plastids was affected. Thus, as in the case of *Ceratopteris* described herein, the duplication of the MTOC appears to be MT independent, but the further movement of the MTOC appears to be MT dependent. Shimamura et al. (2004) investigated a broad range of bryophyte taxa after treatment with oryzalin and found that the γ tubulin always stayed with the putative MTOC (both plastid and more centriolar type), despite the complete elimination of MTs, although the levels of fluorescence at these centers was less than in untreated controls. These data also agree with our observations of γ tubulin being retained on the surface of the blepharoplast despite the absence of MTs on the surface of this MTOC.

The recovery from MT disrupter treatment in plants without recognizable MTOCs shows some variable patterns, however. Cleary and Hardham (1988) discovered that the recovery of MTs after oryzalin treatment did not necessarily reflect a reappearance from known nucleating sites. Rather, sites were more randomly dispersed throughout the cytoplasm, or MTs were re-grown from kinetochore MTs, rather than either cortical (in guard cells or guard mother cells) or nuclear envelope sites that are better established as putative MTOCs in higher plants. It is known that γ-tubulin complexes occur in different sizes in higher plants. Some of the larger ones are clearly membrane associated, such as on the nuclear envelope (Drykova et al. 2003), and may be less dispersed during disrupter treatment than those of a smaller size that might be associated with MTs and/or free in the cytoplasm. Although nothing is known of the size of the γ complex associated with the blepharoplast, the association of γ tubulin with such a large physical structure (and the presence of MPM-2-reactive polypeptides) is indicative of larger complexes in the blepharoplast.

In conclusion, we have found that blepharoplasts of *Ceratopteris*, when denuded of MTs by oryzalin treatment, resemble untreated blepharoplasts, but with small pits; or they may become stretched structures that contain unusual separations of the pericentriolar material and the basal body template cylinders. Blepharoplasts are able to divide in the absence of MTs, but their movement to the poles does not occur. As many as four blepharoplasts (which would be expected from two unsuccessful divisions) can be found within a single spermatogenous cell. Cells with multiple blepharoplasts make sperm cells with multiple motile apparatuses when oryzalin is removed, indicating that the conversion of MTOCs from one type to another is not influenced by their proper separation prior to this division.

Acknowledgements AJB was supported by a USDA-ARS Research Associate position to KCV. Initial studies of oryzalin treatment and recovery were performed by John Hoffman and Neil Durso. These were used to determine appropriate concentrations of oryzalin treatment and recovery time and are gratefully acknowledged here. Culturing of *Ceratopteris* was performed by Lynn Libous-Bailey and Brian Maxwell. H. Joshi generously provided the anti-γ tubulin antibody for use in these experiments. Mention of a trademark, proprietary product or vendor does not constitute an endorsement by the USDA.

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