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Mitotic microtubule development and histone H3 phosphorylation in the holocentric chromosomes of *Rhynchospora tenuis* (Cyperaceae)

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Abstract

In the present work we report the phosphorylation pattern of histone H3 and the development of microtubular structures using immunostaining techniques, in mitosis of *Rhynchospora tenuis* ($2n=4$), a Cyperaceae with holocentric chromosomes. The main features of the holocentric chromosomes of *R. tenuis* coincide with those of other species namely: the absence of primary constriction in prometaphase and metaphase, and the parallel separation of sister chromatids at anaphase. Additionaly, we observed a highly conserved chromosome positioning at anaphase and early telophase sister nuclei. Four microtubule arrangements were distinguished during the root tip cell cycle. Interphase cells showed a cortical microtubule arrangement that progressively forms the characteristic pre-prophase band. At prometaphase the microtubules were homogeneously distributed around the nuclear envelope. Metaphase cells displayed the spindle arrangement with kinetochore microtubules attached throughout the entire chromosome extension. At anaphase kinetochoric microtubules become progressively shorter, whereas bundles of interzonal microtubules became increasingly broader and denser. At late telophase the microtubules were observed equatorially extended beyond the sister nuclei and reaching the cell wall. Immunolabelling with an antibody against phosphorylated histone H3 revealed the four chromosomes labelled throughout their entire extension at metaphase and anaphase. Apparently, the holocentric chromosomes of *R. tenuis* function as an extended centromeric region both in terms of cohesion and H3 phosphorylation.

Introduction

The centromere/kinetochore complex is one of the most fundamental and well differentiated regions of the eukaryotic chromosome. The centromere, composed by repetitive DNA associated with a particular variant of H3 histone, displays a differentiated chromatin fibre arrangement, and plays an essential role in the maintenance of the two sister chromatids joined together until the anaphase onset (Sumner, 2003). On the other hand, the kinetochore comprises a relatively small flat or ball-like proteinaceous structure, localized just over the centromeric region of monocentric chromosomes. It functions as an anchorage region for spindle microtubules and it is indirectly involved in spindle related chromosome movements during mitosis and meiosis (Maney et al., 2000).

Two fundamental types of kinetochores have been described: the localized kinetochore, present in monocentric chromosomes, and the dispersed kinetochore of holocentric or holokinetic chromosomes. The former is common to most eukaryotes, whereas the latter is found in several

invertebrate groups and in a very few groups of plants (Sumner, 2003). The presence of holocentric chromosomes in unrelated taxa suggests that the phenomenon of holocentricity has occurred several times during eukaryotic evolution.

In plants, holocentric chromosomes have been reported only in two closely related families, Cyperaceae and Juncaceae, in the genus *Drosera* (but not in any other member of the family Droseraceae), in one of the subgenera of *Cuscuta* (Convolvulaceae) and in two other isolated species: *Chionographis japonica* Maxim. (Liliaceae) and *Myristica fragrans* Houtt. (Myristicaceae) (Malheiros, De Castro & Câmara, 1947; Flach, 1966; Tanaka & Tanaka, 1977; Pazy & Plitmann, 1994; Sheikh, Kondo & Hoshi, 1995; Vanzela, Guerra & Luceño, 1996; García 2001).

Since the vast majority of species present localized kinetochores, the occasional appearance of holocentric chromosomes is best explained by independent events of replacement during the evolution of distant groups. Noteworthly, the two kinds of kinetochores are surprisingly similar, both at the molecular and the ultrastructural level. Although the holocentric chromosomes exhibit a wide range of kinetochore structures, they are often described as trilaminar, similar to most monocentrics, but with the trilaminar plate extended over the whole or most of the length of the chromosome (Albertson & Thomson, 1982; Goday, Ciofi-Luzzatto & Pimpinelli, 1985; González-García, Benavente & Rufas, 1996b). Several specialized proteins, including the histone CENP-A (HCP-3), which substitutes the histone H3 in the centromeric chromatin, has been found dispersed through the whole chromosomes of *Caenorhabditis elegans* (Dernburg, 2001), the only model organism with holocentric chromosomes.

Moreover, holocentricity seems to be associated with two other important features. Firstly, species with holocentric chromosomes present characteristic meiotic configurations associated with inverted meiosis (Malheiros, De Castro & Câmara, 1947; Hughes-Schader & Schrader, 1961; Albertson & Thomson, 1993). More recently, it has been described a change in the mitotic pattern of the post-translational phosphorylation of aminoacids 10 and 28 of histone H3 (Gernand, Demidov & Houben, 2003). In plants with monocentric chromosomes, histone H3 phosphorylation is restricted or deeply concentrated at the pericentromeric region, but it was found throughout the whole holocentric chromosomes of *Luzula luzuloides* (Madej, 1998; Gernand, Demidov & Houben, 2003). In both plants and animals the phosphorylation of histone H3 occurs only during the transition from prophase to anaphase and it seems to be associated with chromosome condensation and with the maintenance of sister chromatid cohesion (Manzanero et al., 2000). Most interesting, the same H3 phosphorylation pattern of *L. luzuloides* mitotic chromosomes is found in chromosomes of vertebrates and invertebrates with localized kinetochore. Furthermore, the dispersed pattern of histone H3 phosphorylation is found in both meiotic divisions of animals but only in the first meiotic division of plants with monocentric chromosomes, whereas in the second meiotic division histone H3 phosphorylation is restricted to the pericentromeric region (Kaszás & Cande, 2000; Manzanero et al., 2000).

Regarding to the possible existence of centromeric sequences in holocentric chromosomes, Haizel et al. (2005), using the rice 155 bp centromeric tandem repeat sequence (RCS2) as a probe, isolated and characterised a 178 bp tandem sequence repeat (LCS1) from *Luzula nivea*. Using fluorescent *in situ* hybridisation experiments it was shown that there are at least five large clusters of LCS1 sequences distributed at heterochromatin regions along each of the 12 chromosomes of *L. nivea*. The centromeric antibody Skp1 colocalises with these heterochromatin regions and with the LCS1 sequences. This suggests that the LCS1 sequences are part of regions which function as centromeres on these holocentric chromosomes.

In spite of the differences between monocentric and holocentric plant chromosomes, no major difference has been reported in relation to both the interaction of microtubules with kinetochores and the development of the spindle apparatus (Bokhari & Godward, 1980; Albertson & Thomson, 1982; Maddox et al., 2004). Furthermore, the large differences in the development of microtubular structures during mitosis of animals and plants as well as between some specialized plant tissues (Wasteneys, 2002) are fairly established and well documented.

In the present work we report the phosphorylation of histone H3 and the development of microtubular structures observed during the

mitosis of *Rhynchospora tenuis* Vahl., a Cyperaceae with holocentric chromosomes and the lowest chromosome number ever found ($2n = 4$). Its mitotic chromosome behaviour and other structural aspects were previously described by Vanzela, Guerra and Luceño (1996); Vanzela, Cuadrado and Guerra (2003).

Material and methods

Plant material

Plants of *Rhynchospora tenuis* were collected from the locality of Porto de Galinhas (Ipojuca, state of Pernambuco, Brazil) and cultivated in small pots, producing actively growing root tips during many months.

DAPI staining

Some root tips were fixed in a fresh solution of ethanol–acetic acid (3:1, v/v) for a few hours and stored at −20°C. They were washed in distilled water (3 × 10 min), digested in the enzyme solution, and squashed in a drop of 45% acetic acid. The coverslip was removed and the cells were stained with 2 µg/ml DAPI and analysed as described before.

Immunostaining

This procedure was made following the technique described by Manzanero et al. (2000). Non-pretreated root tips were fixed for 45 min in freshly prepared 4% (w/v) paraformaldehyde solution in a microtubule stabilizing buffer (MTSB: 50 mM PIPES, 5 mM $MgSO_4$, 5 mM EGTA, pH 6.9), washed for 45 min in MTSB and digested at 37°C for 1 h and 30 min in a mixture of 2.5% (w/v) cellulase 'Onozuka R-10' and 2.5% (w/v) 'Pectolyase Y-23' dissolved in MTSB. Root tips were then washed for 15 min in MTSB and gently squashed in a drop of MTSB. After freezing in liquid nitrogen, coverslips were removed and the slides were transferred immediately into MTSB.

Blocking was performed for 30 min in 3% BSA (w/v), 0.1% Triton X-100 in MTSB at room temperature. The anti-alpha-tubulin antibody (Amersham) was diluted 1:50 with 3% BSA in MTSB. After overnight incubation at 4°C and washing for 15 min in MTSB, the slides were incubated in FITC-conjugated anti-mouse antibody (Dianova) diluted 1:25 with 3% BSA in MTSB for 3 h at room temperature.

When simultaneous immunostaining of phosphorylated histone H3 was required, the primary antibody was a polyclonal rabbit antiserum that specifically recognized histone H3 phosphorylated at Ser 10 (Upstate Biotechnology, USA). It was diluted 1:300 in 3% BSA in MTSB. After 16 h incubation at 4°C and washing for 15 min in MTSB, slides were incubated in rhodamine-conjugated anti-rabbit IgG (Dianova) diluted 1:200 in MTSB, 3% BSA for 3 h at room temperature.

After final washes in MTSB, the preparations were counterstained with 2 µg/ml DAPI (4′,6-diamidino-2-phenylindole) and mounted in Vectashield H-1000. The best cells were acquired with a Leica DMLB microscope equipped with a Cohu CCD camera and the Leica QWin software and the images were optimized for best contrast and brightness with Adobe Photoshop 6.0. Some slides were studied under a confocal microscope Leica TCS-SP2-AOBS-UV.

Results

The mitotic cycle was first analysed in ethanol–acetic fixed root tips, since after paraformaldehyde fixation the cytoplasm was harder and the separation between anaphase or telophase chromatids was limited. The chromosome complement of *R. tenuis* showed two large and two small chromosomes (Figure 1a), as previously described by Vanzela, Guerra and Luceño (1996). The three most important features concerning the holocentric nature of these chromosomes were: a) the absence of constrictions in prometaphase and metaphase chromosomes; b) the anaphase separation of sister chromatids parallel to each other without any indication of a particular point of localized tension, and c) anaphase and early telophase sister nuclei with highly conserved chromosome positioning, showing perfectly duplicated images. The four chromosomes were observed always curved without keeping any determined position in relation to each other, both in prometaphase and anaphase plates. Rod-shaped metaphase chromosomes were never observed. Figure 1b shows an exceptionally well spread

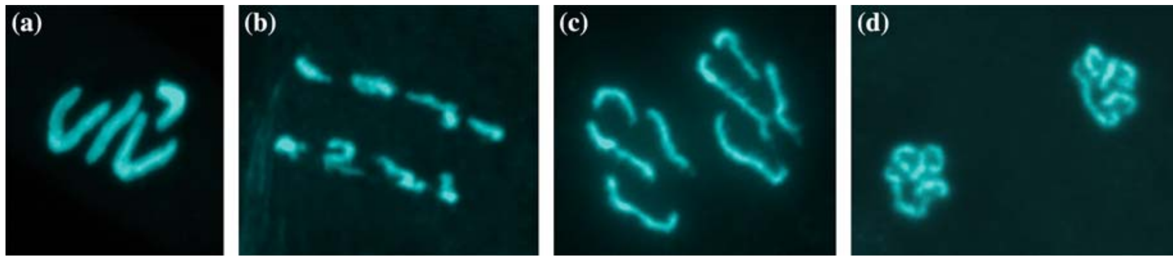

Figure 1. DAPI stained mitotic chromosomes of *Rhynchospora tenuis* and their typical spatial distribution in prometaphase (a), in mid anaphase (b, c) and in later anaphase (d) cells.

anaphase with each chromatid pair individualized and in a clear parallel migration. The anaphase plates (Figure 1c, d) also displayed the same chromosome position observed in the prometaphase plates.

The immunolabelling with the antibody against phosphorylated histone H3 revealed that during the transition from mid-prophase to mid-anaphase the four chromosomes were uniformly labelled throughout their entire extension, reaching the strongest labelling during metaphase (Figure 2). The mitotic nuclei of other phases were always unlabelled (Figure 2b). Four microtubule arrangements were distinguished during the root tip cell cycle of *R. tenuis*: 1) cortical microtubule; 2) preprophase band (PPB); 3) metaphase–anaphase spindle, and 4) phragmoplast. Interphase cells showed a cortical microtubule arrangement, forming a box-like structure. The microtubules were distributed roughly parallel to each other and to the longest cell axis (Figure 3a–c). Prophase nuclei, from very early stages to most advanced

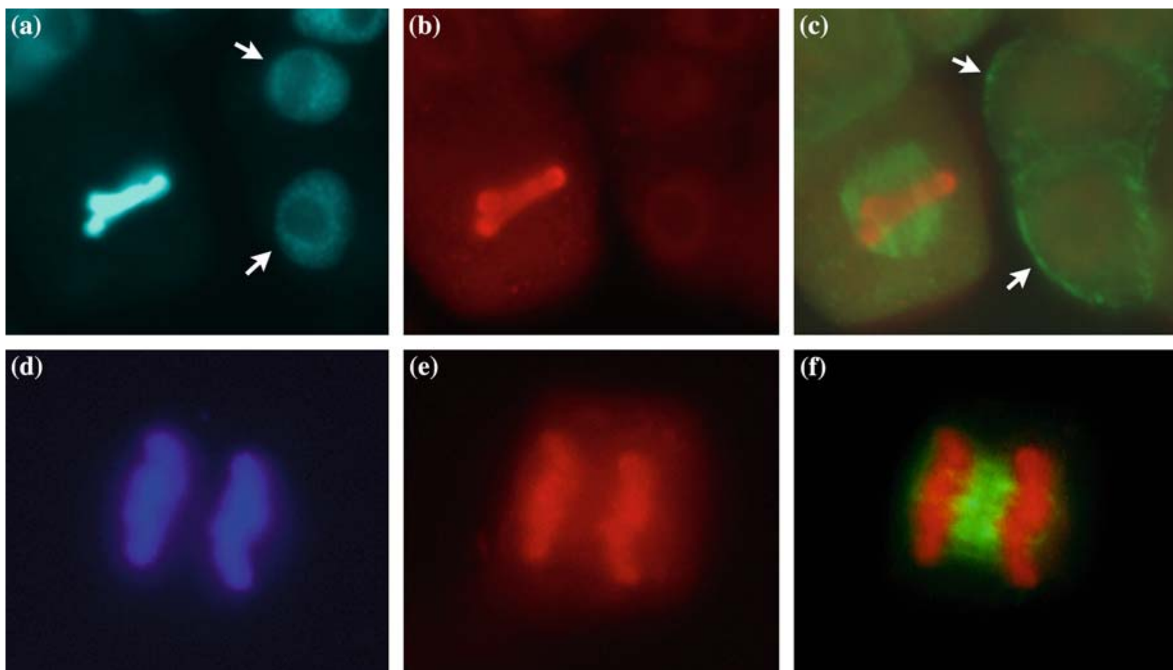

Figure 2. Phosphorylation of histone H3 during different cell stages. a, d. DAPI stained (blue) metaphase, interphase (a) and anaphase (d) cells. b, e. Immunolabelling with the antibody against phosphorylated histone H3 conjugated with TRITC (red). Only the metaphase and anaphase chromosomes were stained. c, f. The same cells showing the distribution of microtubules immunostained with FITC (green) merged with the phosphorylated histone H3 images (red). Arrows in ‘a’ and ‘c’ indicate unlabelled interphase nuclei.

ones, showed the characteristic pre-prophase band, building a broad, brilliant ring around the equator of the nucleus (Figure 3d, e). At prometaphase the microtubules were homogeneously distributed around the chromosomes (Figure 3f). Metaphase cells displayed the spindle arrangement with slightly convergent and poorly defined polar ends (Figure 3g). During this phase, it can be observed that the microtubules are attached to the chromosomes at a tight longitudinal zone, apparently along the whole chromosome. Analysis of metaphase plates at different focal planes showed kinetochore microtubules through the entire chromosome extension. Brighter bundles of microtubules can often be observed in one or two chromosomal regions (Figure 3j–q).

At anaphase the kinetochoric microtubules become progressively shorter, in a very fast way, whereas bundles of interzonal microtubules, parallel to each other and to the division axis, became increasingly broader and denser (Figures 2e, 3h). Most mid and late anaphase cells did not display kinetochoric microtubules but only interzonal ones, although in some mid anaphase cells short kinetochoric microtubules were also visible. The interzonal microtubules and microtubule bundles began and ended closely but not directly in contact with the anaphase or telophase chromosomes. They looked transversally interrupted in a typical phragmoplast differentiation, except at the early anaphase. At late telophase the microtubule bundles of phragmoplast were observed equatorially extended beyond the sister nuclei and reaching the cell wall (Figure 3i).

Discussion

The mitotic cycle analysis of DAPI stained cells confirmed the previous data from Vanzela, Guerra and Luceño (1996) showing that *R. tenuis* presents typical holocentric chromosomes, lacking any constriction at metaphase and each individual chromatid pair migrating always parallel to each other. The duplicated images observed in sister anaphase plates suggest that the whole metaphase plate is equatorially split into two halves with only minimal changes, or no changes at all, in chromosome positioning. Such a duplicated image is only possible if the forces acting over sister chromatids are equally distributed through the entire chromosome complement, as it would be expected in true holocentric chromosomes.

No other reference was found describing the chromosome distribution in anaphase plates with holocentric chromosomes, although the photographs presented by Tanaka and Tanaka (1977) show perfectly duplicated chromosome images of anaphase and telophase cells in *Chionographis japonica* ($2n = 24$). On the other hand, holocentric chromosomes are often reported as presenting precocious poleward movement of chromosome ends at meiotic anaphase (see González-García et al., 1996a) and also during the mitotic anaphase of *Luzula* (Malheiros, De Castro & Câmara, 1947) and some Heteroptera (Hughes-Schrader & Schrader, 1961). This orientation, which was not observed in *R. tenuis*, would result in anaphase plates with inverted rather than duplicated images. These opposite findings probably mean that holocentric chromosomes of different species are not identical in their kinetochore span or kinetic activity. Indeed, the span of the kinetochore in relation to the length of the chromosome is very variable among holocentric chromosomes of different plant species (Braselton, 1971; Bohkari & Godward, 1980) and this variation could account for the differences in anaphase migration and chromosome distribution.

The development of mitotic spindle apparatus in *R. tenuis* followed the general pattern of other plant species with certain variations. Usually, the polar ends of the spindle in plants are not as convergent as in animals and they may be even less evident in plants with holocentric chromosomes like *R. tenuis* and *Luzula luzuloides* (Madej, 1998). This has been attributed to technical constrains of fragile microtubule ends (Hughes-Schrader & Schrader, 1961) or cell type variation (Brown & Lemmon, 2000). However, localized kinetochores did not seem to have this problem. The spindle of holocentric chromosomes has to be able to pull a large plate instead of a localized area as in monocentric chromosomes and since it pulls the plates parallel to each other the pole ends cannot be as convergent as in monocentric chromosomes. Broad spindle ends have also been indirectly observed in other plant species with holocentric chromosomes (Malheiros, De Castro & Câmara, 1947; Tanaka and Tanaka, 1977; Madej, 1998).

The kinetochoric microtubules of *R. tenuis* become progressively shorter soon after the anaphase movement begins and practically disappear

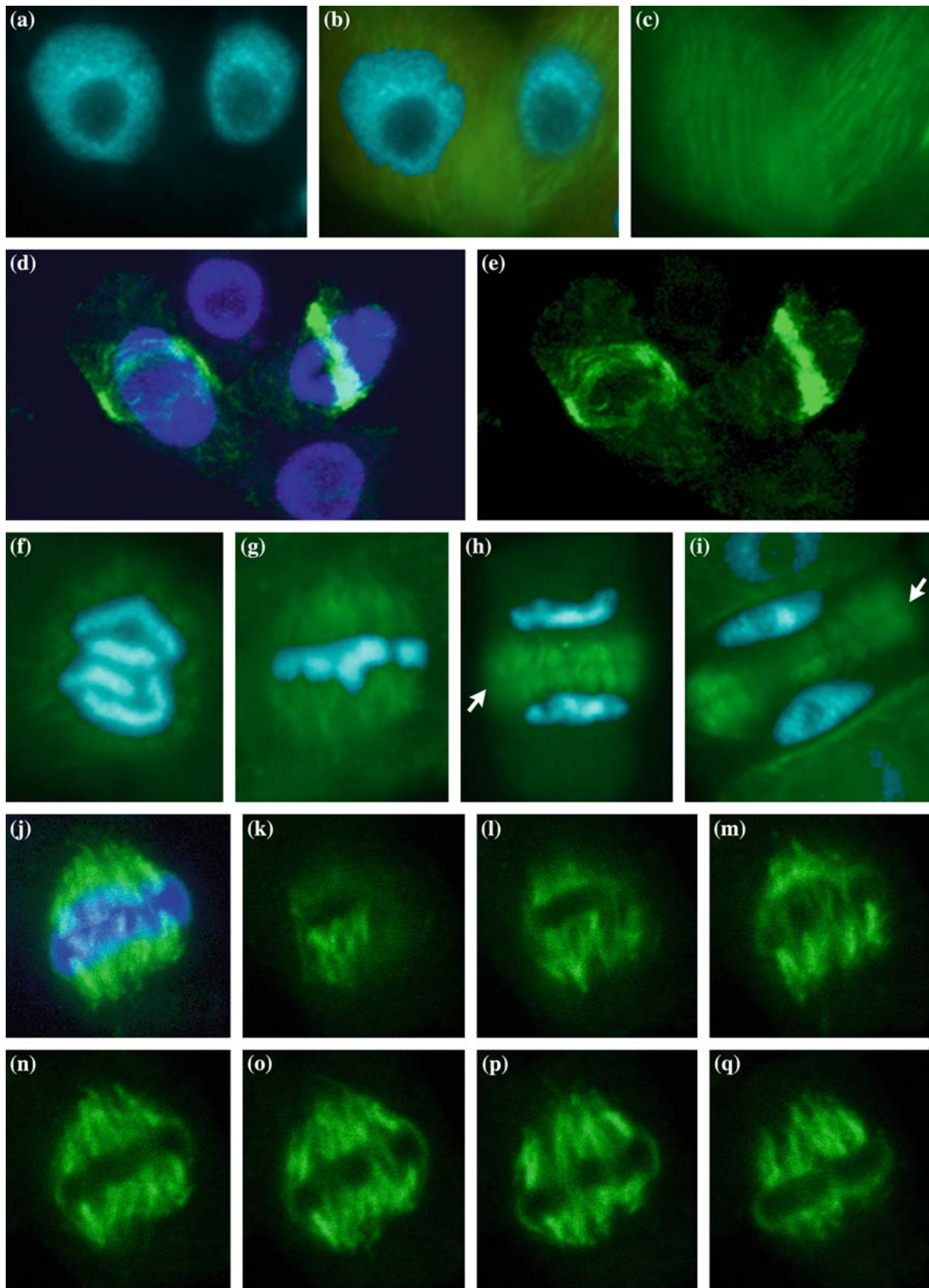
(a)
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Figure 3. Distribution of microtubules during the mitotic cell cycle. a–c. Intact interphase cells with cortical microtubules. d, e. Prophases displaying pre-prophase band. f. Polar view of a prometaphase with microtubules around the chromosomes. g. Metaphase with spindle microtubules. h, i. Anaphase (h) and telophase (i) with interzonal microtubules and phragmoplast. j. Overlay projection of a metaphase showing microtubules attached to the whole chromosome length. k–q. Selected serial plains of the same metaphase (j) showing the distribution of microtubules in individual chromosomes (one chromosome is focused in k and l and two others are best focused in q). Arrows indicate the phragmoplast in 'h' and 'i'.

at mid anaphase. At the same time, the interzonal microtubules arise as the dominant structures, culminating with the formation of the phragmoplast at telophase. The interzonal microtubules, with the polar ends expanding parallel to each other like terminal plates of a cylinder, seem to be related to the parallel chromosome movement and chromosome distribution in duplicated anaphase images. However, no chromosome contact or any other direct evidence of the importance of these microtubules to the anaphase movement was found. Even so, there is an obvious difference in the spindle pole and interzonal microtubules development in plants with holocentric versus localized kinetochores, as for example, the anaphase spindle in rye (Manzanero et al., 2000) and in *Carex* (Brown & Lemonn, 2000). Furthermore, in *Caenorhabditis elegans* the embryonic mitotic separation of anaphase chromatids seems to occur through an anaphase B mechanism (movements from the poles away from each other) whereas the anaphase A, in which the chromatids are pulled in opposite directions, seems to be absent (Oegema et al., 2001). We hypothesize that a similar mechanism accounts for the movement of the mitotic anaphase plates of *R. tenuis* to the poles, with or without the direct involvement of the interzonal microtubules.

Analyses of optical sections of confocal microscopy suggest that metaphase chromosomes are curved rather than rod-like structures (see also Malheiros, De Castro & Câmara, 1947). The microtubules seem to be attached to the metaphase chromosomes along their entire length at a tight median zone, as observed by Bokhari and Godward (1980) in *Luzula nivea*. Brighter bundles of microtubules are observed in metaphase and anaphase cells. However, since every single bundle can be observed transversally in successive focal planes, it more probably represents an optical artefact caused by L or S shaped chromosomes. Apparently, non-pretreated metaphase chromosomes of *R. tenuis* always display such a shape instead of the rod-shaped chromosomes of pretreated metaphases. In metaphase or anaphase plates, flattened L or S shaped chromosomes may have one or two regions pointing to the observer. Such regions might include a larger number of microtubules at the focal plane, seeming denser than the adjacent regions. Furthermore, no sign of localized tension was detected at any point of the chromosomes with DAPI staining. However, an uneven distribution of microtubules through the longitudinal extension of the chromosome cannot be discarded.

In plants with monocentric chromosomes the phosphorylation of histone H3 occurs only at the pericentromeric region at metaphase and anaphase of mitosis (Houben et al., 1999; Manzanero et al., 2000; Manzanero et al., 2002). On the contrary, the results of the present work show that the holocentric chromosomes of *R. tenuis* appear highly phosphorylated along their whole length in mitotic metaphase and anaphase. This result is consistent with the results reported by Madej (1998) and Gernand, Demidov and Houben, (2003) in *Luzula luzuloides*.

Histone H3 phosphorylation at serine 10 and serine 28 has been related both with chromosome condensation along the chromosome mitotic cycle and sister chromatid cohesion at mitosis and meiosis (Kaszás & Cande, 2000; Manzanero et al., 2000; Gernand, Demidov & Houben, 2003). In plants with monocentric chromosomes, during the first meiotic division, when sister chromatids are hold in a more intimate contact, the entire condensed bivalents are highly phosphorylated, whereas in the second division the H3 phosphorylation is restricted to pericentromeric regions, similar to mitotic chromosomes (Manzanero et al., 2000). The observation that in both *Rhynchospora* and *Luzula* the mitotic chromosomes show high levels of phosphorylation along their entire length, together with the fact that whole sister chromatids of holocentric chromosomes are hold tightly paired until the end of metaphase, let us to hypothesize that these two mitotic events are in some way causally related. Hence, the entire length of holocentric chromosomes would be functioning

as an extended centromeric region both in terms of cohesion and H3 phosphorylation. As discussed by Manzanero et al. (2000) the correlation between sister chromatin cohesion and phosphorylation of H3 is not perfect because both processes neither begin nor end simultaneously. For example, sister chromatids separate during anaphase of mitosis without observable dephosphorylation of histone H3 until telophase. However, keeping this association in mind may be fundamental to understand the role of this widespread histone modification.

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