INTRODUCTION

Microcystin-LR (MCY-LR) is a member of a toxic cyclic hBenefit: The elimination of certain cyanobacterial species (Carmichael, 1992; Codd et al., 2005). For example, populations of *Phragmites australis* (common reed), a well-known aquatic macrophyte, can be affected by the toxin. In reed tissue cultures, we have demonstrated that it alters growth as well as histological and cellular organization (Mañé et al., 2007, 2009). MCY-LR is a potent and specific inhibitor of type 1 and 2A serine–threonine protein phosphatases (PP1 and PP2A). In contrast to a significant number of toxins, MCY-LR inhibits these two types of phosphatases with equal potency (MacKintosh and Diplascite, 2003). PP1 and PP2A inhibitors alter the phosphorylation state of proteins playing an important role in cell-cycle regulation (Smith and Walker, 1996; Stals et al., 2000). Due to its biochemical effects, MCY-LR is potentially a useful tool in the study of plant cell cycle regulation.
tool in eukaryotic cell cycle research: an important research topic has been the study of the active role of protein phosphatases in the regulation of mitosis. These enzymes influence the activity of proteins that regulate cytoskeletal and chromatin dynamics during cell division (Trinkle-Mulcahy and Lamond, 2006).

In mammalian cells, it is known that MCY-LR induces microtubule (MT) disruption in mitotic and interphase cells, accompanied by altered chromatin organization in dividing cells (Lankoff et al., 2003; Gács et al., 2009). Such studies are also worth carrying out in plant cells, as the type and regulation of their MT organization differs from animal cells, for example with regard to cortical microtubules, preprophase bands (PPBs) and phragmoplasts as well as the absence of centrioles leading to different mitotic spindle organization as compared with animal cells (Baskin, 2000). MCY-LR is known to inhibit metaphase–anaphase transition in Tradescantia stamen hair cells (Wolniak and Larsen, 1992), but no detailed studies have been made on related changes in MT organization and/or histone phosphorylation. Therefore, understanding the mechanisms involved in such alterations in plant cells needs further investigation.

Histone H3 is an essential component of nucleosomes. It is subject to post-translational modifications. These modifications are thought to serve as marks for transcriptional regulation as well as the timing of chromatin dynamics during interphase, mitosis and meiosis. These signals are generally termed ‘the histone code’ (Prigent and Dimitrov, 2003). In recent decades, H3 phosphorylation at N-terminal Ser and Thr residues was intensively studied in eukaryotic cells. In animal cells, histone H3 phosphorylation is essential for chromatin condensation and therefore transcriptional regulation (Jiang et al., 2004). It is also involved in the regulation of sister chromatid cohesion and segregation (Manzanero et al., 2002; Pérez-Cadahia et al., 2009). In plants, phosphorylated histone H3 (at Ser10 and Ser28) is localized in the pericentromeric region of metaphase chromosomes, while the core centromere complex contains mainly an H3 variant, CENP-A (Schroeder-Reiter et al., 2003).

Due to its small chromosome number (2n = 2x = 12), large chromosomes, relatively short generation time and ease of culture under laboratory conditions, Vicia faba (broad bean) is a widely used model system for plant cell biology and plant genetics research. This includes the study of mitotic chromatin and MT organization and dynamics (Olszewska et al., 1990; Manzanero et al., 2002; Polit and Kaźmierczak, 2007).

Our previous studies of long-term MCY-LR exposure of non-synchronized Phragmites australis root tip meristematic cells have shown that low concentrations of the toxin induced an increase of mitotic activity as well as of early and late mitosis indices. These alterations were accompanied by the formation of aberrant spindles and phragmoplasts as well as altered sister chromatid segregation. This raised the question of whether MCY-LR induces the arrest of cells in certain mitotic phases or just changes the speed of those phases, but allows cells to exit M phase (Máthé et al., 2009). The principal aim of the present study was to answer this question in the V. faba model system and to look for connections between the altered timing of mitosis and the appearance of abnormally dividing cells, when both PP1 and PP2A are inhibited. A widely used method for the study of the timing of mitotic phases is cell synchronization. V. faba proved to be a good model system in this respect (Olszewska et al., 1990; Doležel et al., 1999). In the present study, for a better understanding of altered mitosis induced by inhibition of protein phosphatase, besides the study of mitotic chromatin and MT organization, we looked for changes in the phosphorylation state of histone H3. This is, to our knowledge, the first study looking at the effect of MCY-LR on histone H3 phosphorylation in plant cells and its relationship with toxin-induced mitotic alterations.

**MATERIALS AND METHODS**

**Purification of MCY-LR**

MCY-LR purification was performed according to Kós et al. (1995) with slight modifications. The initial step of extraction with acetone acid was replaced with 80 % (v/v) methanol extraction after repeated freezing–thawing of centrifuged Microcystis aeruginosa cells, a method widely used for the purification of microcystins (Harada et al., 1999). After concentrating with a rotary evaporator at 40 °C (Büchi Rotavapor-R), the extracts were resuspended with 5 mM Tris-HCl, pH 7.5 (Tris base; Sigma-Aldrich, Budapest, Hungary), followed by ion-exchange chromatography on DEAE cellulose (DE-52, Whatman), desalting with Waters Sep-Pak® cartridges according to Kós et al. (1995) and purity checking by high-performance liquid chromatography and the capillary electrophoresis methods described by Vasas et al. (2004). The purity of toxin was ≥95 %.

**Plant material and MCY-LR treatments**

Seeds of broad bean (Vicia faba convar. faba ‘Lippói’) were surface sterilized with 10 % (v/v) commercial bleach, followed by three washes with sterile ion-exchanged water. For long-term MCY-LR treatments, seeds were soaked for 24 h in sterile water in the dark and germinated for 5 d on Murashige-Skoog (MS) medium supplemented with Gamborg’s vitamins and 0·8 % (w/v) Difco-agar (Lawrence, KS, USA) (Murashige and Skoog, 1962; Gamborg et al., 1968), until shoots reached a length of 20 ± 2 (s.e.) mm and main roots had a length of 15 ± 2 mm prior to adding of toxin. Culture conditions for pre-germination and MCY-LR treatments were: 14/10-h light/dark photoperiod with a photon fluence rate of 100 μmol m⁻² s⁻¹ in the light period and a temperature regime of 22 ± 2 °C (light)/18 ± 2 °C (dark). MCY-LR treatments were performed on 5 mL of the same culture medium, but without agar and lasted for 6 d. The toxin concentration range used was 0·5–20 μg mL⁻¹ (0·5–20·1 μM MCY-LR). Samples for histochemistry and immunohistochemistry were taken 4 h after the start of light period. For short-term toxin treatments, seeds were pre-germinated for 10 d, until lateral roots reached a length of 10–15 mm. Lateral roots of seedlings were synchronized with 2·5 mM hydroxyurea (HU; Sigma-Aldrich) for 16 h, according to Doležel et al. (1999). Treatments with 1 and 10 μg mL⁻¹ MCY-LR were performed on 10 mL of liquid.
culture medium at continuous dim light of 3 μmol m⁻² s⁻¹ and a temperature of 20 ± 1 °C, started immediately after HU washout and lasted for 30 h. For cytological analyses, samples were taken at regular time intervals (0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 27, 30 h).

Histochemistry, immunohistochemistry and the analysis of mitotic figures

For labelling of MTs and chromatin, a modification of the procedure of Zhang et al. (1992) was followed (Mathé et al., 2009). For the simultaneous labelling of cells for phosphohistone H3 Ser10 (p-H3 Ser10), we followed the principle of indirect immunohistochemical labelling of Manzanero et al. (2002). Vicia faba lateral root tips (at least 5–6 root tips per treatment) were fixed with 4 % (v/v) formaldehyde in phosphate-buffered saline (PBS) for 16 h, followed by cryosectioning. Sections 10–15 μm thick were subjected to direct MT labelling with a Cy3-conjugated anti-β tubulin antibody (Sigma-Aldrich) according to the manufacturer’s instructions applied to plant cells: sections were washed with PBS and cells were permeabilized by 10 min treatment with PBS containing 0⋅5 % (v/v) Triton X-100 (Reanal). After three washing steps, microtubules were labelled for 16 h with the antibody diluted with PBS containing 1 % bovine serum albumin (BSA; Sigma-Aldrich). Labeling for p-H3 Ser10 was performed with a 50-fold diluted rabbit primary antibody (cat. No. 05-817, Upstate, Lake Placid, NY, USA) at 4 °C, for 12 h. After two washing steps, secondary antibody was added at a 100-fold dilution (Alexa 488-conjugated goat anti-rabbit IgG; Molecular Probes, Eugene, Oregon, USA) for 4 h at 37 °C. Immunohistochemical labelling was followed by washing with PBS and staining with 3 μg mL⁻¹ 4′,6′-diamidino-2-phenylindole (DAPI; Fluka, Buchs, Switzerland) for 40 min. Sections washed with PBS were mounted on microscopic slides in antifade buffer containing 0⋅1 % (w/v) p-phenylenediamine (Sigma-Aldrich) in 90 % (v/v) glycerol (Reanal, pH 8). Microscopic examinations were made with an Olympus Provis AX-70/A fluorescence microscope. Excitation filter parameters were as follows: 540–580 nm for Cy3, 450–480 nm for Alexa 488 and 320–360 nm for DAPI. For better visualization of intracellular structures, a Zeiss LSM 510 confocal laser scanning microscope was used. Excitation wavelengths were 543 nm (Cy3), 488 nm (Alexa 488) and 351/364 nm (DAPI). Fluorescence emission was detected through 560–615-, 505–550- and 385–470-nm band-pass filters. Images were taken in multitrack mode. A Plan-Apochromat oil immersion objective (NA 1.4) was used and image stacks of 1.0–1.5-μm-thick optical sections were taken.

During the detection of mitotic structures, we examined the meristematic zone of lateral root tips, but excluded the quiescent zone and cell lineages giving rise to rhizodermal and vascular tissues. Total mitotic indices, early and late mitosis indices, the percentage of mitotic anomalies (chromatin and MT anomalies out of total mitotic cells) as well as micronucleus formation were examined in at least 30 sections of 5–6 lateral root tips per treatment. Those sections were previously subject to histochemical and immunohistochemical labelling as described above.

Western blot analysis of p-H3 Ser10

Total protein was extracted from lateral root tips of V. faba treated for 6 d with MCY-LR, according to Beyer et al. (2009). Deep-frozen tissues were extracted with 62.5 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol (Sigma-Aldrich) and 0⋅5 % (v/v) protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) at 95 °C and centrifuged at 20 000 g. For assay of protein content, samples from supernatants were precipitated with 100 % acetone (Reanal) and resuspended in 0⋅25 mM NaOH (Reanal). Protein content was measured according to Bradford (1976). The remaining supernatants were used for Western blotting. Proteins (16 μg per well) were separated on 10 % polyacrylamide gels by SDS-PAGE according to Laemmli (1970). Proteins were blotted onto nitrocellulose membranes (Bio-Rad, Heidelberg, Germany) using a Bio-Rad electrobloot system. For membrane blocking, 5 % non-fat dried milk in TBS containing 0⋅1 % Tween 20 was used. After blocking, the membranes were washed with the same buffer but without dried milk and incubated with the same anti-p-H3 Ser10 primary antibody as for immunohistochemistry, and for anti-β-tubulin (rabbit primary antibody; Upstate) for 3 h. This was followed by application of the horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Sigma-Aldrich) for 90 min at room temperature.

Immunoreactions were detected by enhanced chemiluminescence (ECL). ECL reagent was from Thermo Scientific (Waltham, MA, USA). The dilution of antibodies and ECL detection were according to the manufacturer’s instructions.

Data analysis

All experiments were repeated at least five times. For mitotic indices and counting of mitotic anomalies, standard errors were calculated and results were plotted with the use of Sigma Plot® 10.0 software. In the case of long-term MCY-LR treatments, the significance of differences as compared with controls was analysed with one-way analysis of variance (ANOVA), followed by the multiple comparison procedure. For all statistical methods, toxin effects were considered to be significant as compared with controls, when P < 0.05.

RESULTS

The effects of long-term MCY-LR treatment on mitotic structures and mitotic activity in non-synchronized V. faba lateral root tip meristems

Long-term MCY-LR treatments lasted for 6 d. Labelling of MTs with an anti-β-tubulin antibody and of chromatin with DAPI revealed normal organization of mitotic apparatus in control cells. Metaphase and anaphase cells had normal, bipolar spindles with the proper alignment and segregation of chromatids (Fig. 1A, B). When cells entered telophase, spindles were replaced by phragmoplasts that expanded by late telophase/cytokinesis. Sister chromatids were segregated normally (Fig. 1C, D). Treatment with MCY-LR induced several mitotic alterations, including: (1) disrupted or monopolar metaphase spindles accompanied by the hypercondensation
and misalignment of chromosomes (Fig. 1E, E'); (2) the formation of multipolar spindles at early anaphase and the appearance of lagging chromosomes at late anaphase (Fig. 1F, F'); and (3) disruption of phragmoplasts (Fig. 1G, arrows) and the unequal distribution of chromatids during telophase/cytokinesis (Fig. 1G, arrowhead). Altered organization of PPBs or prophase chromatin was not detected (data not shown).

Histone H3 phosphorylation at Ser 10 was not detectable in interphase cells. Labelling appeared in the pericentromeric regions of prophase chromosomes (Fig. 2A). In metaphase and anaphase, pericentromeric regions were strongly labelled, but weak labelling could be detected in chromosome arms as well. p-H3 Ser10 label was weak, nearly undetectable in telophase (Fig. 2A). In contrast, MCY-LR induced a strong and uniform labelling of whole metaphase chromosomes, including chromosome arms. This strong label persisted in early telophase and faded away only at late telophase/cytokinesis (Fig. 2B). Western blotting of lateral root tip protein extracts revealed the phosphorylation of histone H3 at Ser 10, as a clear band appeared at 17 kDa (Fig. 2C). Band intensity increased gradually at 1–10 μg mL⁻¹ MCY-LR. At the highest toxin concentration used (20 μg mL⁻¹), band intensity decreased as compared with treatments with 10 μg mL⁻¹ MCY-LR, but it was still higher than in controls (Fig. 2C).

Concomitant labelling of MTs and phospho-histone H3 showed normal metaphase spindles and histone H3 phosphorylation in controls (Fig. 2D). The effects of MCY-LR

**FIG. 1.** The effects of long-term (6 d) microcystin-LR (MCY-LR) treatments on chromatin and microtubule organization in mitotic *Vicia faba* cells. Lateral root tip meristematic cells were stained with DAPI while microtubules were labelled with a Cy3-conjugated anti-β-tubulin antibody: merged images are also shown. Control cells in metaphase (A), anaphase (B) with normal chromatin and spindle organization. Control cells in telophase (C) and late telophase/cytokinesis (D) with normal chromatin and phragmoplast organization. (E) Metaphase cell exposed to 5 μg mL⁻¹ MCY-LR, with hypercondensed chromatin and disrupted spindle. (E') Metaphase cell exposed to 5 μg mL⁻¹ MCY-LR, with misaligned chromosomes (arrowhead) and monopolar spindle. (F) Early anaphase cell exposed to 0.5 μg mL⁻¹ MCY-LR, with multipolar spindle. (F') Late anaphase cell exposed to 10 μg mL⁻¹ MCY-LR treated with lagging chromosome (arrowheads). (G) Late telophase/cytokininetic cell exposed to 0.5 μg mL⁻¹ MCY-LR with a micronucleus (arrowhead) and phragmoplast disruption (arrow). Scale bars: 10 μm.
treatments were: (1) increased p-H3 Ser10 label, but spindle organization remained normal (Fig. 2E); (2) normal p-H3 Ser10 label with disrupted spindle (Fig. 2F); and (3) an increase of p-H3 Ser10 label concomitantly with the formation of disrupted metaphase spindle (Fig. 2G).

In control cells, histochemical labelling of chromatin with DAPI revealed normal chromatid segregation during telophase with the formation of intact nuclei in interphase (Fig. 3A). MCY-LR treatments induced the formation of lagging chromosomes at late anaphase (Fig. 3B), the formation of micronuclei in telophase (Fig. 3C) and the persistence of micronuclei in interphase cells (Fig. 3D). The sporadic occurrence of micronuclei in controls (Fig. 4D) could not be related to the formation of laggards.

MCY-LR stimulated mitosis as compared with controls. Highest mitotic activity was detected at low (0.5–1 μg mL⁻¹) toxin concentrations. The effect of 1 μg mL⁻¹ MCY-LR was significant (Fig. 4A). We found similar stimulatory effects, when distinct mitotic phases were analysed. The highest percentage of prophase was at 1 μg mL⁻¹ MCY-LR, while the highest metaphase index was at 10 μg mL⁻¹ MCY-LR. These effects were significant (Fig. 4B). Concerning late mitosis, all toxin concentrations induced a slight increase of anaphase frequency. A significant increase of telophase index was detected at 0.5–5 μg mL⁻¹ MCY-LR and 0.5 μg mL⁻¹ MCY-LR induced the strongest increase (Fig. 4B).

We did not detect altered mitosis in control cells. Most mitotic anomalies occurred at all toxin concentrations examined, but their maximal occurrence was at 10 μg mL⁻¹ MCY-LR, which coincided with the maximum of altered metaphase frequency (Fig. 3C). The highest frequency of altered anaphases was at 5–10 μg mL⁻¹ MCY-LR, and the highest ratio of altered telophases was at 0.5 μg mL⁻¹ MCY-LR, but still remained high at 1–20 μg mL⁻¹ MCY-LR (Fig. 4C). All toxin concentrations increased the occurrence of micronuclei significantly (Fig. 4D). In controls, 13.33 ± 2.1 % of lateral roots contained micronuclei in their apical meristems, compared with 60 ± 5.77 % at 10 μg mL⁻¹ MCY-LR and 86.66 ± 6.66 % at 20 μg mL⁻¹ MCY-LR.

The effect of short-term MCY-LR treatments on mitotic structures and mitotic activity in synchronized V. faba lateral root tip meristems

In synchronized control cells the changes of histone phosphorylation pattern during mitosis were similar to those...
observed in non-synchronized cells (Figs 2A and 5A–D). There was no detectable p-H3 Ser10 label in interphase cells (data not shown). Ten hours after HU washout, cells started to divide (Fig. 6A, B). This coincided with the appearance of p-H3 Ser10 label at the pericentromeric regions of prophase chromosomes (Fig. 5A, A'). The intensity of this label increased in metaphase, and weak H3 phosphorylation could be detected in chromosome arms, too (Fig. 5B). This weak label of chromosome arms persisted in anaphase and gradually decreased in telophase (Fig. 5C, D). MCY-LR had a clearly detectable effect on this phosphorylation pattern at both concentrations used (1 and 10 μg mL⁻¹). The toxin did not alter cell division, and meristematic cells completed two mitotic cycles in the time period examined (30 h) (Fig. 6A). In contrast to controls, where the duration of mitosis was 12 h, cell division lasted for 6 h in the presence of 10 μg mL⁻¹ MCY-LR. Mitotic peaks were at 14 and 24 h, and the first peak showed a higher mitotic index as compared with the mitotic peak of controls (Fig. 6A). The progression of early mitosis had similar characteristics to total mitotic activity (Fig. 6B). At 18 h after HU washout, the occurrence of pro- and metaphases was still high as compared with controls (Fig. 6B, arrow). Note that at 18 h, the frequency of cells in late mitosis was higher than in controls at 18 h (Fig. 6C). A cell population had its late mitosis peak at 22 h (Fig. 6C, arrow).

The effect of 10 μg mL⁻¹ MCY-LR was markedly different from that of the low toxin dose. It stimulated entry into mitosis as well, but it did not delay its completion. Instead, it accelerated cell division, and meristematic cells completed two mitotic cycles in the time period examined (30 h) (Fig. 6A). The progression of late mitosis showed the maximal occurrence of early mitosis (prophase and metaphase) was similar to total mitotic activity (Fig. 6B). The maximal occurrence of late mitotic cells (anaphase and telophase) was at 18 h after HU washout, but the duration of mitosis was longer than in controls: its maximal intensity was at 14–18 h and it was completed only at 24 h after HU washout (Fig. 6A). The progression of early mitosis was similar to total mitotic activity (Fig. 6B). At 18 h after HU washout, the occurrence of pro- and metaphases was still high as compared with controls (Fig. 6B, arrow). Note that at 18 h, the frequency of cells in late mitosis was higher than in controls at 18 h (Fig. 6C). A cell population had its late mitosis peak at 22 h (Fig. 6C, arrow).

At short-term toxin treatments of synchronized *V. faba* cells, altered MT organization was not detected (data not shown).

As mentioned above, control meristematic cells started mitosis 10 h after HU washout. The maximum frequency of mitotic cells was at 14 h, and cells completed mitosis at 22 h. There was a cell population that started a second mitotic cycle 27 h after HU washout (Fig. 6A). The progression of early mitosis (prophase and metaphase) was similar to total mitotic activity (Fig. 6B). The maximal occurrence of late mitotic cells (anaphase and telophase) was at 18 h after HU washout, but a cell population entered late mitosis at 27 h as well (Fig. 6C). MCY-LR induced significant alterations of mitotic activity of synchronized cells. At 1 μg mL⁻¹, it accelerated entry into mitosis (cells started to divide at 8 h after HU washout) but the duration of mitosis was longer than in controls: its maximal intensity was at 14–18 h and it was completed only at 24 h after HU washout (Fig. 6A). The progression of early mitosis was similar to total mitotic activity (Fig. 6B). At 18 h after HU washout, the occurrence of pro- and metaphases was still high as compared with controls (Fig. 6B, arrow). Note that at 18 h, the frequency of cells in late mitosis was higher than in controls at 18 h (Fig. 6C). A cell population had its late mitosis peak at 22 h (Fig. 6C, arrow).

**DISCUSSION**

**MCY-LR alters mitotic activity and induces mitotic anomalies in *V. faba***

Long-term MCY-LR exposure of *V. faba* lateral root meristematic cells increased their general mitotic activity as well as the percentage of cells in early and late mitotic phases (Fig. 4). This modulation of cell division activity was somewhat

![Fig. 3. Long-term (6 d) microcystin-LR (MCY-LR) exposure induces micronucleus formation in mitotic *V. faba* cells. Chromatin was labelled with DAPI. (A) Control cells in telophase (t) and interphase (i). (B–C) Treatment with 10 μg mL⁻¹ MCY-LR; formation of micronucleus (arrows); (B) late anaphase, lagging chromosome; (C) late telophase, the beginning of chromatin decondensation and micronucleus formation. (D) Interphase cells; one cell contains a micronucleus. Scale bars: 10 μm.](image-url)
similar to that observed in *Phragmites australis* root meristems that were exposed to low MCY-LR concentrations over the long term. However, in the latter plant, the toxin inhibited mitosis at high concentrations (Ma´ the´ et al., 2009), which was not observed in *V. faba* (Fig. 4A). The highest percentage of prophases occurred at a lower concentration of MCY-LR (1 µg mL$^{-1}$) compared with metaphases (10 µg mL$^{-1}$). The highest percentage of anaphases occurred at 20 µg mL$^{-1}$ MCY-LR and that of telophases was at 0.5 µg mL$^{-1}$ (Fig. 4B). Thus, a stronger inhibition of protein phosphatases is required for the increase of metaphase and anaphase indices, but in general, a wide toxin concentration range is capable of stimulating early and late mitosis. As we have shown that similar MCY-LR concentrations induce increases in the number of meta- and anaphases (although this was significant only for metaphases), we predicted that MCY-LR alters metaphase–anaphase transition. Indeed, short-term toxin treatments of synchronized *V. faba* lateral root meristematic cells revealed that MCY-LR did not arrest cells in early or late mitosis. Instead, at lower concentrations (1 µg mL$^{-1}$) it accelerated the onset of mitosis and delayed metaphase–anaphase transition of certain cell populations: the time necessary for the completion of early mitosis was longer than in controls, but cells entered into late mitosis thereafter (Fig. 6). There was a cell population that entered into anaphase later than the remaining meristematic cells (Fig. 6C, arrow). The toxin induced the inhibition of mitotic exit of those cells, but they completed mitosis as well. Higher concentrations of MCY-LR (10 µg mL$^{-1}$) accelerated cell cycle: while control cells completed only one mitotic cycle within 30 h, the toxin induced the completion of two cell division cycles (Fig. 6).

What could be the cause of MCY-LR-induced mitotic stimulation? As a protein phosphatase inhibitor, the toxin may influence mitotic activity through the modulation of mitogen-activated protein kinase (MAPK) activities in animal cells (Gehringer, 2004). Cyclin-dependent kinase and MAPK isotypes are important cell cycle regulators not only in yeast and animal cells, but also in plant cells. They play an important role in the phosphorylation of proteins involved in the regulation of chromatin dynamics during cell division (Polit and Kaz´ mierczak, 2007) and of microtubule-associated proteins (MAPs) that influence MT dynamics during the plant cell cycle (Smertenko et al., 2006). The involvement of cell cycle regulators in these processes raises the idea that MCY-LR-induced changes of mitotic activity and alterations of mitotic chromatin and MT organization in *V. faba* are interrelated processes. The study of chromatin and microtubule structures in long-term toxin treatments underlined this relationship: (1) the toxin concentration (10 µg mL$^{-1}$) required for the highest frequency of metaphases and of cells with...
Control MCY-LR

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hypercromosed metaphase chromosomes and altered spindles coincided (Figs 2B and 4); (2) the highest telophase index and the highest frequencey of altered telophases was at the same, low MCY-LR concentration (0.5 μg mL⁻¹) (Fig. 4B, C); and (3) altered metaphase–anaphase transition was accompanied by the formation of multipolar, multinuclear and monopolar spindles, and altered chromatin segregation (Figs 1 and 4). Evidence for the presence of altered transition is that the highest frequency of altered metaphase- and anaphase occurs at similar MCY-LR concentrations (5–10 μg mL⁻¹, Fig. 4C).

In mammalian cells, the inhibition of protein dephosphorylation induces similar alterations: the inhibition of metaphase–anaphase transition accompanied by the formation of abnormal spindles, leading to altered chromatin segregation (Vandré and Wills, 1992; Lankoff et al., 2003; Bonness et al., 2006). Endothall and okadaic acid (OA), drugs that inhibit PP2A more potently than PP1, arrested alfalfa and tobacco cells in prophase (Zhang et al., 1992; Ayaydin et al., 2000). MCY-LR did not have such an effect in V. faba, indicating that the equal inhibition of PP1 and PP2A may lead to different mitotic changes than in the case of preferential inhibition of PP2A.

The formation of micronuclei or micronucleus-like structures can be induced by three mechanisms: (1) apoptotic fragmentation of nuclei (Lytvyn et al., 2010), (2) chromosome breakage (Leme and Marin-Morales, 2009) and (3) incomplete sister chromatid segregation with the formation of lagging chromosomes (Ma et al., 1995). For MCY-LR-treated V. faba roots, the first two mechanisms are unlikely, as: (1) during apoptosis, fragmentation of the nucleus is preceded by its blebbing and leads to the formation of multiple satellite nuclei (Michalakis et al., 2005) – we detected single micronuclei but not nuclear blebbing (Fig. 3); and (2) our cytogenetic observations did not show MCY-LR-induced chromosomal breakage (data not shown). Our data support the third mechanism of micronucleus formation: (1) the formation of laggards was followed by the appearance of micronuclei under both short- and long-term MCY-LR treatments (Figs 3, 4 and 6D); and (2) in synchronized meristems, 1 μg mL⁻¹ MCY-LR stimulated the formation of micronuclei in a similar manner to the increase of the percentage of cells in late telophase – there were two cell populations in telophase as well as increased micronucleus formation (Fig. 6C, D, arrows). This indicates a correlation between the alteration of late mitotic activity and micronucleus formation. Altered telophases and micronuclei appeared at all MCY-LR concentrations examined in long-term treatments.

**MCY-LR induces histone H3 hyperphosphorylation at Ser10 and altered chromatid segregation**

Similarly to our observations, Poli and Kazmierczak (2007) found that OA induced premature onset and acceleration of mitosis, accompanied by hypercondensation of prophase and metaphase chromosomes in V. faba root meristems. OA-induced incomplete sister chromatid segregation led to unequal distribution of chromatids at cell poles and the formation of lagging chromosomes. The authors correlated this to the onset of mitosis before completion of heterochromatin replication in S phase. In the present study we show that MCY-LR-induced chromosome hypercondensation and abnormal chromatid segregation occur concomitantly with the hyperphosphorylation of histone H3 (Figs 2 and 5). In V. faba, detectable H3 phosphorylation occurs only in mitotic cells (this study), whereas in Xenopus, MCY-LR induces histone H3 hyperphosphorylation in interphase cells (Murnion et al., 2001). In control mitotic cells, we showed that the dynamics of histone H3 phosphorylation at Ser10 follows the general pattern of plant cells (Manzanero et al., 2002; Houben et al., 2007): in interphase, it remains at levels below detection; in prophase, mainly pericentric regions are labelled for p-H3 Ser10 and the intensity of this label increases in metaphase. Label gradually decreases in intensity during late anaphase and telophase. In contrast, MCY-LR treatment leads to a strong labelling of metaphase chromosomes, not only at pericentric regions but in chromosome arms as well. In contrast to control cells, this label remains intense in late mitosis (Figs 2 and 5). It is known that the phosphorylation state of plant histone H3 and its centromeric variant, CENP-A/CENH3, at distinct amino acid residues at the N-terminal region play an important role in chromatin condensation, cohesion and subsequent segregation: their phosphorylation level must be higher in prophase and especially in metaphase, and decreases in anaphase and telophase (Schroeder-Reiter et al., 2003; Zhang et al., 2005). Normal chromosome condensation and the dephosphorylation of histone H3 can be altered by inhibition of both PP1 and PP2A in yeast and animal cells. The inhibition of PP2A by fostriecin increases the overall level of histone H3 phosphorylation, as revealed by autoradiography of mouse cell extracts (Guo et al., 1995), whereas Hsu et al. (2000) showed that PP1 is involved in dephosphorylation at Ser10. Cantharidin induces histone H3 hyperphosphorylation at Ser10 in plant chromosomes (Manzanero et al., 2002). As MCY-LR-induced histone H3 hyperphosphorylation occurred...
with the hypercondensation of metaphase chromosomes in *V. faba* (Figs 2B and 5F, F'), one may assume that there is a relationship between these two phenomena. Although in plants the correlation between histone phosphorylation and chromosome condensation appears not to be as close as in animal cells (Manzanero *et al.*, 2002; Houben *et al.*, 2007), hyperphosphorylation appears concomitantly with strong sister chromatid cohesion and chromosome hypercondensation in mitotic and meiotic plant cells (Kaszás and Cande, 2000; Manzanero *et al.*, 2002). In the presence of MCY-LR, hyperphosphorylation of histone H3 in highly condensed metaphase chromosomes occurred concomitantly with their circular/rosette-like arrangement in a significant number of cells (Fig. 5F'). Similar metaphase rosettes were observed in cantharidin-treated mitotic barley cells (Manzanero *et al.*, 2002). Although this rosette-like distribution seems to be normal for many eukaryotic cells, it is far more visible at high condensation rates of chromosomes (Nagele *et al.*, 1998). The protein phosphatases responsible for the regulation of phosphorylation state of histone H3 are less well known in plants. MCY-LR can provide useful data in this regard.

Incomplete chromatid segregation could be induced by high concentrations of endothall that inhibited both PP1 and PP2A. At lower concentrations, where PP2A inhibition was predominant, it did not induce segregation anomalies, but it increased the percentage of cells in telophase (Ayaydin *et al.*, 2000). MCY-LR (which inhibits equally PP1 and PP2A) increases the percentage of cells in late mitosis as well, both in *Phragmites australis* and in *V. faba* (Mathé *et al.*, 2009; Figs 4B and 6C). Concomitantly, it induces altered sister chromatid segregation, even at lower concentrations. Accordingly, it appears that the increase of telophase frequency was caused mainly by the inhibition of PP2A. Cantharidin is another drug that inhibits mainly PP2A activity. Although it delays metaphase–anaphase transition, it barely induces the formation of lagging chromosomes in plant cells, even though it induces histone H3 hyperphosphorylation at Ser10 in the entire chromosome. It is possible that cantharidin did not increase chromatid cohesion, indicating that H3 hyperphosphorylation induced by PP2A inhibition by itself is not sufficient to induce excessive cohesion (Manzanero *et al.*, 2002; Houben *et al.*, 2007). Thus, PP2A activity is required for histone H3 dephosphorylation and the regulation of entry into and exit from late mitosis in plant cells. The role of PP1 in these processes cannot be excluded. PP2A is less involved in the regulation of chromatid segregation. Besides H3, other proteins that are subject to dephosphorylation by PP1 are involved in the correct assembly of centromeric structures regulating sister chromatid cohesion. MCY-LR-induced H3 hyperphosphorylation at Ser10 can contribute to the strong cohesion of sister chromatids at the centromeric regions. Such changes in centromeric structures are involved in abnormal chromatid segregation and delay the onset of late mitosis. MCY-LR-induced mitotic chromatin alterations might contribute to a better understanding of the mechanisms involved in the regulation of metaphase–anaphase transition.

**FIG. 6.** The effect of short-term microcystin-LR (MCY-LR) exposure on mitotic activity and the formation of micronuclei of synchronized *V. faba* lateral root tip meristematic cells. (A) Mitotic indices. (B) Early mitosis (prophase and metaphase) indices showing the delay of early mitosis in the presence of MCY-LR (arrow). (C) Late mitosis (anaphase and telophase) indices. Arrow shows a second peak of late mitosis in the presence of 1 μg mL⁻¹ MCY-LR. (D) MCY-LR-induced increases of micronuclei numbers in lateral root tip meristems (1 μg mL⁻¹ MCY-LR, arrows; 10 μg mL⁻¹ MCY-LR, arrowhead).

MCY-LR-induced micronucleus formation can be related to alterations of mitotic MT organization as well

Endothall induced chromosome hypercondensation in prophase, interfered with PPB formation, induced the formation of multipolar prometaphase spindles, inhibited entry into metaphase and altered phragmoplast maturation in alfalfa...
cells (Ayaydin et al., 2000). By contrast, long-term MCY-LR exposure did not induce prophase anomalies, but it did influence spindle and phragmoplast organization in V. faba (Figs 1 and 4). These findings highlight the similarities and differences between the role of PP1 and PP2A in the correct organization of mitotic microtubules. Under long-term MCY-LR treatments, we detected the formation of disrupted, multipolar or monopolar spindles in metaphase and early anaphase, but this appeared not to influence the normal shortening of kinetochore fibres at late anaphase. However, the development of abnormal metaphase and early anaphase spindles occurred concomitantly with chromosome misalignment and was sufficient for the formation of lagging chromosomes (Fig. 1E, E’, F, F’). In human tumorous cells, the formation of multipolar spindles does not allow correct sister chromatid segregation, leading to the formation of aneuploid cells. Abnormal spindle organization is induced by the alteration of MT binding of phosphorylation-dependent MAPs (Venoux et al., 2008).

The formation of monopolar spindles leads to the delay or complete blocking of chromatid movement during anaphase and the formation of micronuclei (Holy, 2002). Under long-term MCY-LR treatments of V. faba meristems, misalignment of metaphase chromosomes was accompanied by monopolar spindles (Fig. 1E’), which could lead to altered chromatid segregation and micronucleus formation. Lankoff et al. (2003) found monopolar spindles and aberrant anaphases in MCY-LR-treated CHO-K1 cells. The present study is the first report of cyanotoxin-induced monopolar spindle formation in plants.

Both hypo- and hyperphosphorylated states of certain MAP65 proteins alter normal MT cross-linking during plant mitosis, leading to altered spindle and phragmoplast formation (Smertenko et al., 2006; Beck et al., 2011). Therefore, MCY-LR-induced altered (disrupted, monopolar or multipolar) spindles and disrupted phragmoplasts (Fig. 1) might be related to the hyperphosphorylation of MAPs important in the regulation of normal mitotic MT assembly.

Based on the above, disruption of metaphase and anaphase spindle organization contributes to the alteration of metaphase–anaphase transition and leads to incomplete sister chromatid segregation and the formation of micronuclei.

MCY-LR-induced histone H3 hyperphosphorylation and altered spindle organization is both consequences of the inhibition of protein dephosphorylation, but they are probably independent processes. High phospho-histone H3 signal was uniformly distributed in chromosomes of long-term MCY-LR-treated cells even when spindle structure was normal (Fig. 2D, E). Similar findings were made by Manzanero et al. (2002) in cantharadin-treated plant cells. By contrast, short-term MCY-LR treatment of synchronized V. faba root tip meristematic cells resulted in sporadic microtubule anomalies, while hyperphosphorylation of histone H3 was regularly observed (Fig. 5). However, under long-term MCY-LR treatments, abnormal chromatid segregation and micronuclear formation are, among several possible factors, a result of concomitant disorganization of the mitotic MT apparatus and the alteration of histone H3 phosphorylation. It has been suggested that histone H3 phosphorylation at Ser10 contributes to the regulation of correct spindle MT attachment to kinetochores (Hirot a et al., 2005), and it is therefore possible that long-term MCY-LR treatments alter this kinetochore–H3–MT relationship.

**Conclusions**

MCY-LR, a protein phosphatase inhibitor, has a mitotic stimulatory effect, but alters metaphase–anaphase transition in V. faba root meristematic cells. Due to altered transition to late mitosis, there is an increase in the duration of early mitosis and a delay in the completion of cell division, but this was not accompanied by the total inhibition of mitotic exit. These changes of mitotic activity are accompanied by alterations in chromatin and cytoskeletal organization. The main conclusion of this study is that MCY-LR-induced alteration of sister chromatid segregation has a major role in the formation of micronuclei. We have shown for the first time that (1) toxin-induced histone H3 hyperphosphorylation leads to chromosome hypercondensation and an increase of chromatid cohesion in metaphase that will delay the onset of anaphase and inhibit normal chromatid segregation; and (2) the alteration of mitotic spindle organization contributes to altered chromatid segregation as well. However, we did not find a clear mechanistic relationship between these two types of alterations. We add further data to the idea that protein dephosphorylation plays an active role in the correct organization of mitotic chromatin and cytoskeleton. MCY-LR can be useful in the study of plant cell cycle regulation.

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