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Microcystin-LR, a cyanotoxin, modulates division of higher plant chloroplasts through protein phosphatase inhibition and affects cyanobacterial division

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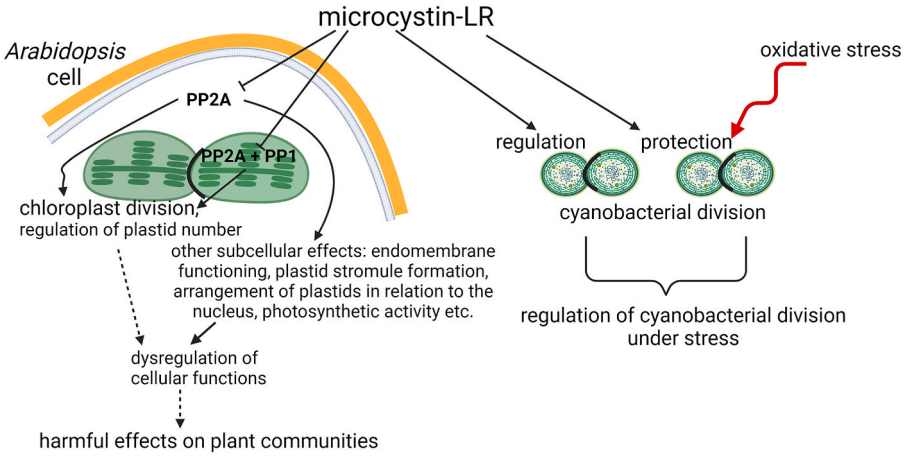
HIGHLIGHTS

- Microcystin-LR (MC-LR) is a harmful cyanotoxin, an inhibitor of protein phosphatases.
- MC-LR alters Arabidopsis plastid division at environmentally relevant concentration.
- Environmental implications of such subcellular alterations in plants are discussed.
- MC-LR also affects cyanobacterial division even during oxidative stress.
- MC-LR's evolutionary function may be the regulation of cyanobacterial division.

GRAPHICAL ABSTRACT



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ABSTRACT

Microcystin-LR (MC-LR) is a harmful cyanotoxin that inhibits 1 and 2A serine-threonine protein phosphatases. This study examines the influence of MC-LR on chloroplast division and the underlying mechanisms and consequences in *Arabidopsis*. MC-LR increased the frequency of dividing chloroplasts in hypocotyls in a time range of 1–96 h. At short-term exposures to MC-LR, small-sized chloroplasts (longitudinal diameters ≤6 μm) were more

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sensitive to these stimulatory effects, while both small and large chloroplasts showed stimulations at long-term exposure. After 48 h, the cyanotoxin increased the frequency of small-sized chloroplasts, indicating the stimulation of division. MC-LR inhibited protein phosphatases in whole hypocotyls and isolated chloroplasts, while it did not induce oxidative stress. We show for the first time that total cellular phosphatases play important roles in chloroplast division and that particular chloroplast phosphatases may be involved in these processes. Interestingly, MC-LR has a protective effect on cyanobacterial division during methyl-viologen (MV) treatments in *Synechococcus* PCC6301. MC-LR production has harmful effects on ecosystems and it may have an ancient cell division regulatory role in stressed cyanobacterial cells, the evolutionary ancestors of chloroplasts. We propose that cytoplasmic (eukaryotic) factors also contribute to the relevant effects of MC-LR in plants.

1. Introduction

Harmful cyanobacterial blooms/harmful algal blooms (HABs) are of increasing concern in the 21st century, occurring worldwide due to climate change and freshwater eutrophication (Paerl, 2009; EC Joint Research European Commission. Joint Research Centre, 2016). Of the cyanobacterial toxins (cyanotoxins) detected in HABs, microcystins (MCs) are the most well-known and are produced by several genera, e.g. *Microcystis*, *Anabaena*, *Planktothrix*, *Lyngbya*. Among them, microcystin-LR (MC-LR) is the most studied congener (Rastogi et al., 2014). MCs are potent inhibitors of type 1 and 2A, serine-threonine protein phosphatases. Moreover, they not only modulate their activities, but increase phosphatase gene expression as a compensatory mechanism. This was demonstrated in MC-LR treated rice and cucumber seedlings (MacKintosh and Diplexcito, 2010; Ma et al., 2023). Since these enzymes are of crucial importance in key eukaryotic biochemical/cellular events (Farkas et al., 2007), it is not surprising that MCs have significant impacts on human/animal/plant and in general, environmental health (see Szigeti et al., 2010; Ujvárosi et al., 2019 for examples). Moreover, MCs are chemically stable, persisting for extended periods in water bodies (Kós et al., 1995; Vasas et al., 2010; Cai et al., 2022; Wei et al., 2023), which further increases the environmental hazard they pose. MCs induce oxidative stress as well in a number of eukaryotic organisms, but the relationship of this effect to protein phosphatase inhibition remains controversial (Máthé et al., 2019).

The environmentally relevant freshwater concentrations of MCs are in the nM - μM (μg L^{-1} – mg L^{-1}) range. The nM range is the most characteristic for freshwater HABs, but reports increasingly show toxin contents in the μM range, not only in tropical, but in temperate waterbodies as well (Máthé et al., 2007; Paerl, 2009; Chia et al., 2022). During laboratory exposures for cellular studies in plants, we have previously found that relatively higher MC-LR concentrations (μM range) induce similar alterations at short-term exposures to lower concentrations (in the nM range) at long-term exposures (e.g. Máthé et al., 2009, 2013).

The work of Omidi et al. (2017) gives an overview of the potential biological functions of MC-LR and MCs in general. These are: (i) the production of MCs offers a competitive advantage for the producing cyanobacteria in relation to other species of phytoplankton communities; (ii) MC production has a protective role against zooplankton grazers. These two ideas may be valid, although many studies show MCs to be ineffective against several phyto- and zooplankton species (iii) MCs are capable of binding peptides (e.g. glutathione). involved in the regulation of oxidative stress, thereby regulating the redox status of the cyanobacterial cell; (iv) they can chelate different metal ions, such as iron, regulating the availability of these chemical elements to cyanobacterial metabolic processes.

The recent work of Zheng et al. (2023) raises the possibility that MC-LR is a factor that down-regulates cell division of the producing cyanobacterium. When the *mcy* gene cluster was expressed in the model strain *Synechococcus* 7942, cell division frequency was altered. The authors proposed that the cyanotoxin molecule was competing with the GTP-binding site of FtsZ, causing the mislocalization of this protein, which is important for the fission of prokaryotic cells. However, there is a lack of prior research on the effects of MCs on plastid division. These statements are interesting in the light of cyanobacterial (endosymbiotic) origin of plastids, because the chloroplast division machinery still has many similarities with that of cyanobacteria. Therefore, it appears worthwhile to perform a more detailed comparative analysis of chloroplast and cyanobacterial division under MC-LR exposure.

Arabidopsis thaliana is an ideal model for studying subcellular alterations induced by the cyanotoxin. MC-LR is effective in this plant at concentrations of 50 nM and above, with most of the changes observed at 1 μM during short-term (4h–24h) exposures (Omidi et al., 2017; Freytag et al., 2021, 2023). In the present study, the 1 μM concentration was found to be suitable for both long- and short-term exposures, so this concentration was used throughout the experiments presented here.

Plastid division is an important process, since it regulates the number of these important organelles of cyanobacterial origin in plant cells (Pyke, 1997; Cheng et al., 2018). Following plastid DNA replication, the fission process is governed by a complex protein machinery, partly of prokaryotic origin, - known as the inner and outer ring complexes. This machinery is characteristic for several plastid types including proplastids, leucoplasts, and chloroplasts. The inner FtsZ 1/2 ring consists of FtsZ proteins of prokaryotic origin, while the outer ring consists of ARC5, a eukaryotic dynamin-like protein. The two rings are connected by diverse proteins, among which ARC6 and PARC6 have homologues only in cyanobacteria (Miyagishima, 2011; Cheng et al., 2018).

The chloroplast phosphoproteome is relatively well studied (Schönberg and Baginsky, 2012). While several aspects of the regulation of division ring complexes are known, little attention has been paid to their dependence on reversible protein phosphorylation or the relevant effects of protein phosphatase inhibitors. MC-LR is known to influence other aspects of structural organization of plastids, such as stromule formation. Stromules are tiny emergencies of the plastid stroma known to be important in the increase of surface for membrane contact sites with other compartments (Barton et al., 2017; Juhász et al., 2023).

Most environmental stresses induce oxidative damage at the cell level (Apel and Hirt, 2004). Methyl viologen (MV, paraquat) is an inducer of oxidative stress by blocking electron transport in chloroplasts and mitochondria. It is often used as a model stressor in several cell types (e.g. Schansker et al., 2005). Therefore, we used MV in the present study to assess whether MC-LR interferes with MV treatments during chloroplast and cyanobacterial division. As previously mentioned, MCs may induce oxidative stress in a variety of organisms.

In light of the above statements, we designed experiments to compare the effects of MC-LR, a protein phosphatase inhibitory cyanotoxin on plastid and cyanobacterial division. We aimed to answer the following questions.

(i) How does MC-LR influence the division of chloroplasts in *Arabidopsis* hypocotyls? What are the possible underlying mechanisms? Does the toxin influence chloroplast-level, cytoplasmic (eukaryotic) or both types of plastid division regulatory processes? Can the effects be related to protein phosphatase inhibition, or does oxidative stress also play a role in the effects of the cyanotoxin?
(ii) How does MC-LR influence cyanobacterial division? Does it modify the inhibitory effect of MV, a reactive oxygen species

(ROS) inducer? Do the effects of MC-LR on chloroplasts show similarities to its effects on cyanobacterial division?

One of our objectives was to answer the question: are the effects of MC-LR on chloroplast division related to any relevant influence of this cyanotoxin on cyanobacteria, the evolutionary ancestors of chloroplasts? To our knowledge, this aspect is not well understood to date. The environmental relevance of our findings will also be discussed.

2. Materials and methods

2.1. The purification of MC-LR

Throughout this study, microcystin-LR (MC-LR) was purified for use in treating Arabidopsis seedlings. *Microcystis aeruginosa* BGSD243 originated from a water bloom in Lake Velencei, (Hungary) was used as the source organism. The purification was performed essentially by the method of Kós et al. (1995) after a significant modification according to Vasas et al. (2004) as optimized to maximal efficiency. In the first step, methanolic extracts were subjected to ion-exchange (DEAE-cellulose chromatography), then further purified by HPLC. Validity was checked using a commercial MC-LR standard (Gold Standard Diagnostics, Westminster, PA, USA), and LC-MS was employed (Máthé et al., 2013; Freytag et al., 2023). The resulting MC-LR preparation was free of contaminants, with a purity of >95%. This preparation was lyophilized, then a stock solution was prepared by taking into account the $\varepsilon = 13.7$ value of the compound (Kós et al., 1995). The concentration of stock was 1 mM (taking into account the MW 994 of MC-LR, this was approximately 1 mg mL^{-1}).

2.2. Plant material and treatments

Arabidopsis thaliana L. Columbia (Col0, wild-type and genotypes bearing fluorescent protein-fusion proteins) ecotype was used for all studies involving plant treatments. For the assay of chloroplast division, the proARC5ARC5:YFP construct (Col0 background) was used as a marker, while Col0 wild-type for TEM, ROS content and protein phosphatase assays. For additional studies of plastid clustering around the nucleus associated ER membranes, combined tpFNR:GFP and RFP-ER constructs of a Col0 as well as *arc5* mutant background were used (Schattat et al., 2011, 2012; Delfosse et al., 2016).

Before cyanotoxin treatments, *Arabidopsis* seeds were surface sterilized and plated onto Difco-agar solidified MS media supplemented with Gamborg's vitamins (Murashige and Skoog, 1962; Gamborg et al., 1968) as described before (Nagy et al., 2018; Freytag et al., 2021). Growth conditions included a 14/10 h photoperiod, 22 ± 2 °C, 60 μmol $m^{-2}s^{-1}$ photon flux density in the light period. Seven – days - old seedlings with well-developed hypocotyls were used for MC-LR treatments on liquid medium according to Freytag et al. (2021, 2023). Briefly, seedlings were transferred to 50-mm petri dishes with sterilized filter papers soaked with constant volumes of the liquid MS media supplemented with Gamborg's vitamins, then treatments started. For treatments, purified MC-LR was used, as specified in Section 2.1. The final cyanotoxin concentration used was 1 μM (this corresponds to 1 μg mL^{-1}), prepared by diluting the stock specified in Section 2.1. with culture medium. This final concentration was determined to be effective, since concentrations below 1 μM only partially influenced chloroplast division, while higher concentrations induced shoot necrosis or were even lethal in Col0 plants. Treatment duration for the analysis of division activities was between 1 and 96 h.

2.3. Microscopy analysis of plant samples

For live cell imaging, whole hypocotyls were analyzed by confocal laser scanning microscopy (CLSM) with the following parameters. For chloroplast division, the YFP signal of proARC5ARC5:YFP plants was detected with a Leica TCS-SP5 CLSM as merged images obtained by excitation with 488 nm Ar and 543 nm He–Ne lasers. Emission parameters were 490–510 nm for GFP, 558–596 nm for RFP and 600–680 nm for chlorophyll autofluorescence. Chloroplast division was also examined with an Olympus Fluoview 1000 CLSM (Olympus, Tokyo, Japan), with excitation parameters: 515 nm for YFP and 633 nm for chlorophyll autofluorescence. Red chlorophyll fluorescence was then digitally pseudo-colored to blue to obtain a better contrast to the fluorescence signal of YFP (for detection of ARC5).

The percentage of chloroplasts showing YFP signals was quantified to examine the time-dependent effects of MC-LR on chloroplast division. Short-term (4h) effects of MV and MC-LR + MV combinations were also examined. At least three hypocotyls, a minimum of twenty cells/hypocotyl per treatment per experiment (for each time point in a given experiment) were examined and at least five repetitions were performed for each treatment.

For the examination of chloroplast size distribution in controls and MC-LR treatments, TEM analysis was performed as described previously (Nagy et al., 2018). The microscopy device used was a Hitachi 7100 transmission electron microscope (Hitachi, Tokyo, Japan). Epidermal cells were excluded from these measurements. Five hypocotyls per treatment were sectioned and 90–140 randomly chosen chloroplasts per hypocotyl were measured for chloroplast length. Only intact images of morphometrically relevant sections (presumably showing full length of the organelle) were used for size measurements. Experiments were repeated three times.

2.4. Quantification of ROS contents in hypocotyls

Following cyanotoxin treatments, living hypocotyls were examined for total ROS content through a two-step (labeling and washing) procedure using 10 μM of 2′,7′-dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, Mo., USA). We followed the method described previously for labeling whole root tissues at pH 7.0 under dark conditions, to maintain stability of the dye (Freytag et al., 2023, based on the protocol presented in Garda et al., 2016). Samples were examined with an Olympus BX43 fluorescence microscope (Olympus, Tokyo, Japan), excitation wavelength range was 450–480 nm. The intensity of fluorescence, an indicator of ROS content, was quantified using the Fiji software, which calculated AIOD (area integrated optical density). Eight to ten hypocotyls were examined per treatment per experiment, and experiments were repeated four times. ROS content was analyzed in hypocotyls of seedlings treated for 4–120 h with MC-LR.

2.5. The assay of protein phosphatase activities

Protein phosphatase assays were essentially performed as described previously (Erdődi et al., 1995; Máthé et al., 2013; Garda et al., 2018). The preparation of enzyme extracts was carried out as follows.

Hypocotyl extracts were prepared with 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 mM EGTA, 0.1 % (w/v) DTT, 0.2 mM PMSF (all from Sigma-Aldrich, St. Louis, Mo., USA) and 0.5 % (v/v) protease inhibitor cocktail (Roche Appl. Sci., Indianapolis, IN, USA).

For the assay of plastid protein phosphatase activities, hypocotyl chloroplasts were isolated by following the Percoll gradient procedure of Cline (1986). Isolated chloroplasts were suspended in a buffer containing 50 mM HEPES/KOH (Sigma-Aldrich), pH 7.5 and 0.33 M sorbitol (Duchefa Biochemie, Haarlem, The Netherlands) and treated with MC-LR for 2 h. For preparation of enzyme extracts, chloroplast suspensions were centrifuged repeatedly with a Digicen 21R centrifuge/swinging bucket rotor (Álvarez Redondo S.A., Madrid, Spain) for 3–5 min at 4 °C, then chloroplasts were resuspended with the phosphatase extraction buffer (see above for whole hypocotyl extracts) and disrupted by sonication (Branson Sonifer 2.50 sonicator, Branson Ultrasonics Corp., Danbury, Co., USA) 1.5 impulse intensity, 30% duty cycle with 3x30 s durations, at 0 °C.

Both hypocotyl and chloroplast extracts were centrifuged with a Beckman Avanti Series centrifuge (Beckman, Brea, Ca., USA) at 13000×*g* for 15 min at 4 °C. Protein content of supernatants was determined by the Bradford (1976) method. The activity assay used ^{32}P-MLC20 (turkey gizzard 20 kDa myosin light chain, prepared at the Department of Medical Chemistry, UD) as a substrate. Specific protein phosphatase activities were expressed as pmol ^{32}Pi released mg protein^{-1}. The separate assay of PP2A and PP1 activities was performed using 2 μM Inhibitor-2 (I-2, prepared at the Department of Medical Chemistry, UD) protein (Dedinszki et al., 2015). Total (PP2A + PP1) as

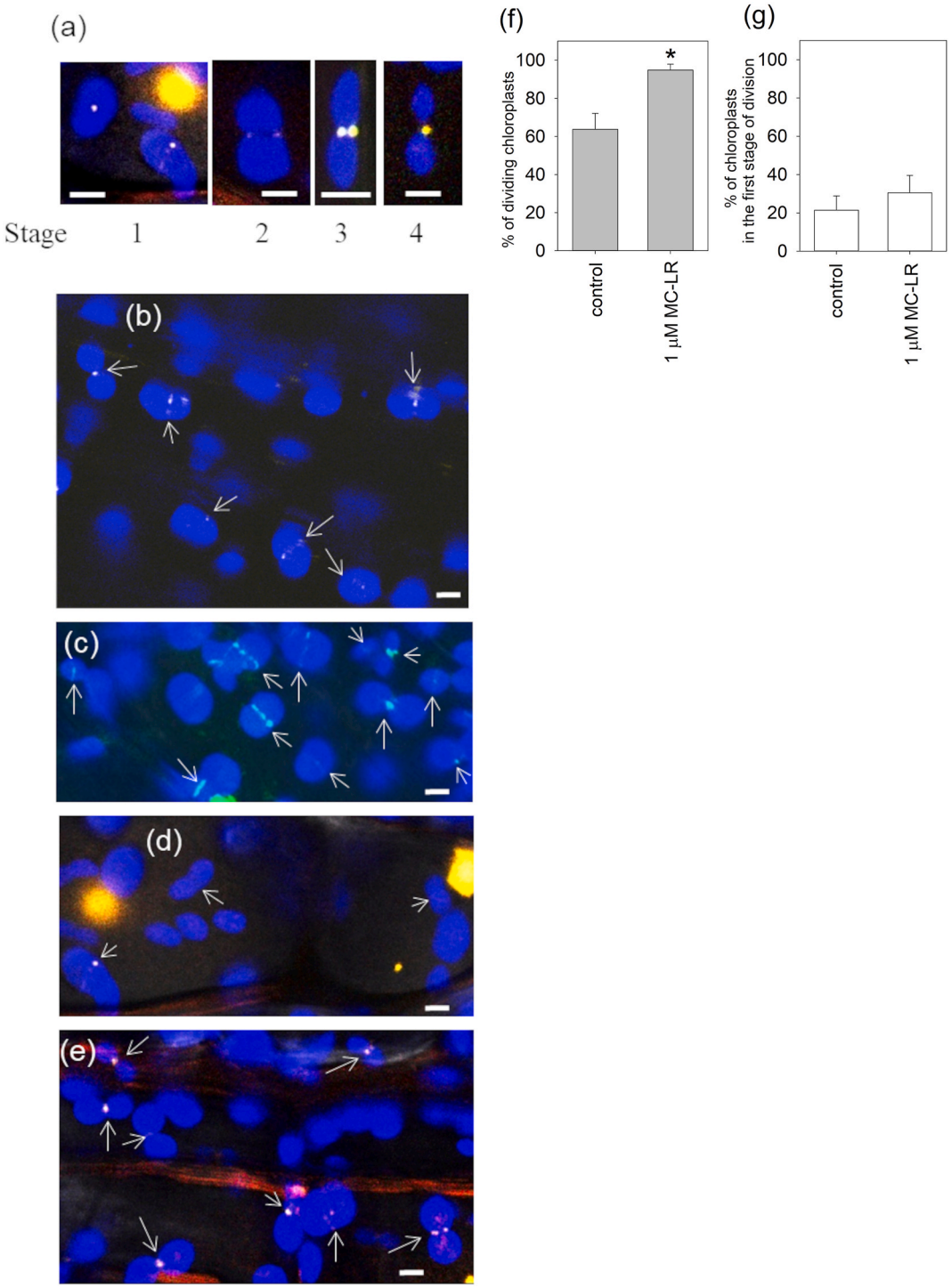

Fig. 1. MC-LR changes the percentage of dividing chloroplasts as detected by the ARC5 signals in hypocotyls of proARC5ARC5:YFP transgenic lines. **(a)** Stages of chloroplast fission during the division process. Yellow: YFP signal; blue: chlorophyll autofluorescence. These stages were stated according to (Miyagishima et al., 2006). **(b)** YFP signals (arrow) in chloroplasts of control hypocotyl cells after 4 h; **(c)** YFP signals (arrows) in chloroplasts of hypocotyl cells exposed to 1 μM MC-LR for 4 h. **(d)** YFP signals (arrow) in chloroplasts of control hypocotyl cells after 4 days; **(e)** YFP signals (arrows) in chloroplasts of hypocotyl cells exposed to 1 μM MC-LR for 4 days. **(f)** short-term (4h) effect of MC-LR on chloroplast division; **(g)** short-term (4h) effect of MC-LR on the percentage of chloroplasts in the first stage of fission. Statistical analysis was performed by *t*-test, *P < 0.05. Scalebars: 3 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

well as distinct PP2A and PP1 activities were measured in 3–6 repetitions for each sample per treatment per experiment and experiments were repeated at least three times.

2.6. The assay of cyanobacterial growth and cell division

The model cyanobacterium *Synechococcus* PCC 6301 was treated with the cyanotoxin as well as methyl-viologen (MV, Sigma-Aldrich) and a combination of these two compounds. For treatments of *Synechococcus* PPC6301 cells, the concentration of MV was carefully chosen. Preliminary experiments were conducted using a wide concentration range of MV to establish that 2 μM MV inhibits growth but is not lethal to 100% of cells, at least for short-term (up to 10 h) treatments. The effective MC-LR concentration was equimolar to MV. Cyanobacterial cell growth was assayed as the number of cells per mL counted with a Bürker chamber. The same approach was used for the counting of cells with visible division/fission activities. Both the number of cells in fission per mL and the cell division index (percentage of cells in fission from total cell population) were examined. Examination was performed using an Olympus BX43 fluorescence microscope. Bright-field facilities were used, but for better visibility, fluorescence facilities were also employed to detect chlorophyll-a autofluorescence by excitation at 530–555 nm. All experiments regarding the single and combined effects of MC-LR and MV on *Synechococcus* PCC6301 were repeated four times.

2.7. Data analysis

Data were plotted by showing the mean ± SE values-with the aid of Systat Sigma Plot 10.0 ® and 12.0 ® software (Systat Software, San Jose, CA). Statistical significances for the differences between controls and treatments were studied using t-tests, except in cases where the normality test failed. In the latter case, the Mann-Whitney Rank Sum test was used. Significant differences were * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. For Fig. 5 (cyanobacterial growth and division), significant differences are indicated by lettercodes, as explained in the figure caption.

3. Results

3.1. The effects of MC-LR on chloroplast division/fission

Stages of chloroplast division/fission were identified according to

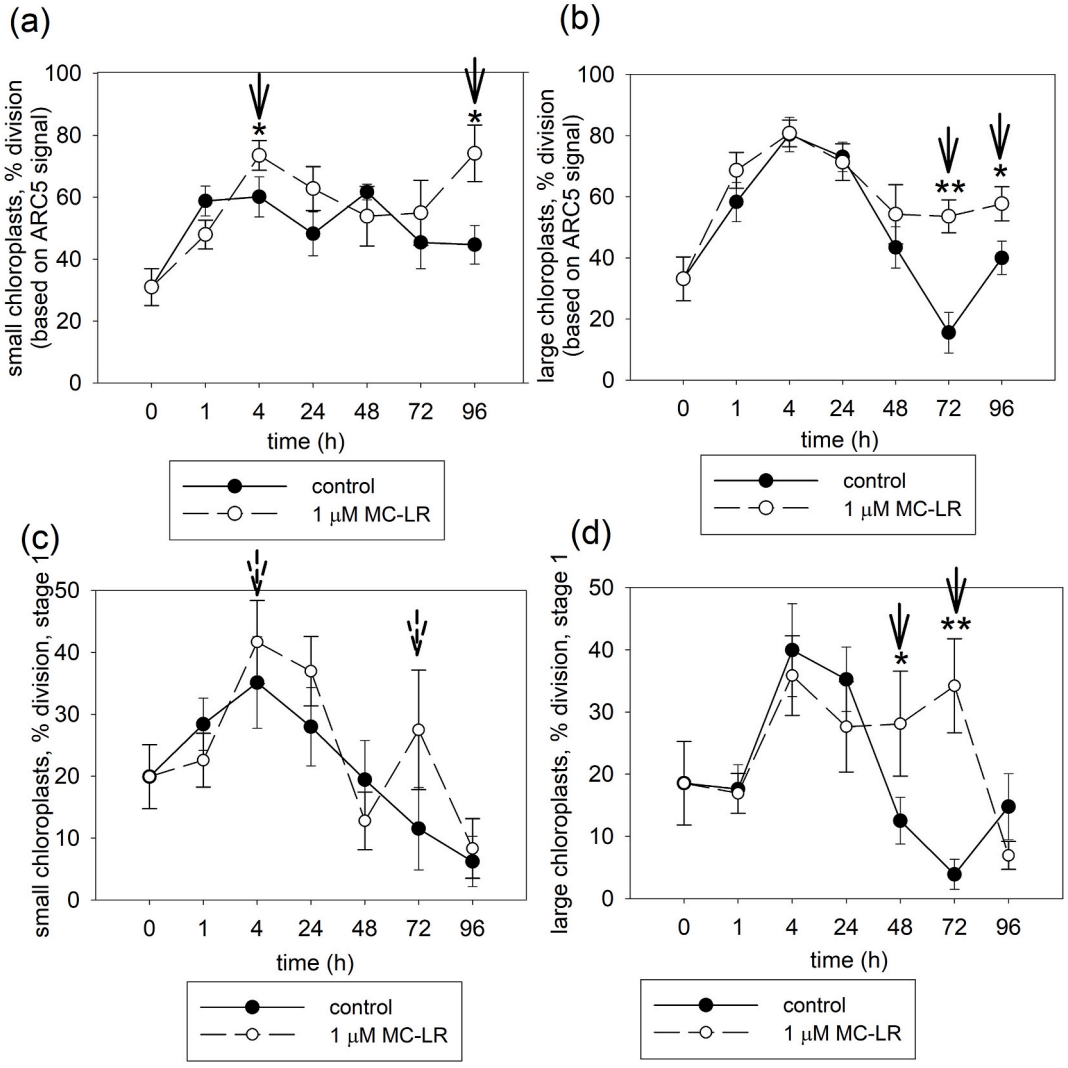

Fig. 2. MC-LR changes the percentage of dividing small and large chloroplasts as detected by the ARC5 signals in hypocotyls of proARC5ARC5:YFP transgenic lines. Time course of the effects of MC-LR on all stages of fission of small (**a**) and large (**b**) chloroplasts. For small chloroplasts, the increasing effect of MC-LR is biphasic, having an early and late effect (arrows in **a**, **c**). Time course of the effects of MC-LR on the frequency of the first fission stage for small (**c**) and large (**d**) chloroplasts showing increases after long-term MC-LR exposures that are significant for large chloroplasts (arrows). Statistical analysis of the differences between controls and treatments at all exposure times was performed by *t*-test and Mann-Whitney Rank Sum tests, *$P < 0.05$, **$P < 0.01$.

the signal of YFP:ARC5 fusion protein in the chlorophyll autofluorescence background (Fig. 1a). The YFP signal was visible in the midplane of chloroplasts, before fission became visible. This was considered stage 1. At stages 2–3, fission was clearly detectable and at stage 4, fission was completed. All these stages were taken into account as division/fission activity. At relatively short-term (4 h) MC-LR exposures (at 1 μM, the effective concentration), there was a noticeable increase of division activity as compared to controls (Fig. 1b and c). At long term exposures (96 h, i.e. 4 days), a similar increasing effect was observed (Fig. 1d and e). We quantified division of the total chloroplast population at 4 h of cyanotoxin exposure. MC-LR induced a significant increase of division activity (Fig. 1f). MC-LR treatment led to a slight, non-significant increase in the percentage of chloroplasts in the 1st stage of division, but in general, this stage was characterized by a relatively low level both for control and treated hypocotyls (Fig. 1g).

A detailed time-course analysis of division activity was conducted for both small-sized (longitudinal diameter ≤6 μm) and large chloroplasts. For small chloroplasts, controls showed division activity at all examined time frames, with the highest frequencies at 1h - 48 h. MC-LR increased division activities in a significant manner at 4h and 96 h of exposure (Fig. 2a, arrows). Concerning stage 1 of division, a 4 h peak was observed for controls, and stage 1 frequency decreased gradually thereafter. MC-LR had a stimulatory effect with peaks at 4 h and 72 h (Fig. 2c, arrows). For large chloroplasts, division peaked at 4 h in controls. MC-LR did not further increase division activity at 4h, but maintained significantly higher division frequency at 72 and 96 h of exposure (Fig. 2b, arrows). A significant toxin-induced increase of stage 1 division frequency was detected after 48 and 72 h of exposure, with no increase at 96 h (Fig. 2d, arrow).

TEM analysis for the size distribution of chloroplasts in hypocotyls of Col0 plants showed that the population of small-sized chloroplasts increased at 48 h and 96 h of cyanotoxin exposure (Fig. 3a–c, arrows), while no relevant changes were observed for 72 h of exposure (Fig. 3b).

3.2. The effects of MC-LR on ROS contents and protein phosphatase activities

DCFH-DA labeling revealed that control hypocotyls had very low levels of total ROS compared to roots of seedlings (compare Figs. 4a to 1 of Freytag et al., 2023). MC-LR did not induce any significant changes in terms of ROS in the examined time range (4h–120h/5 days) (Fig. 4a).

Analysis of total protein phosphatase (PP2A + PP1) activities of whole hypocotyls revealed a slight inhibition at 4 h of cyanotoxin exposure. This inhibition became significant at 24 and 48 h of exposure (Fig. 4b). When PP2A and PP1 activities were analyzed separately, both activities proved to be inhibited in a significant manner, but PP2A activity was primarily affected (Fig. 4b', b"). Two hours of MC-LR exposure of isolated shoot chloroplasts showed that phosphatase activities were strongly and significantly inhibited. This is valid both for total activities and separate PP2A and PP1 activities (Fig. 4c, c', c").

3.3. The effects of MC-LR on the division of Synechococcus PCC6301 cells. Results for combined MC-LR and methyl viologen (MV) treatments of cyanobacteral cells and Arabidopsis seedlings

For a comparative analysis of the effects of MC-LR and MV on the division of cyanobacterial cells and chloroplasts, we chose *Synechococcus* PCC6301, a non-cyanotoxin producing model cyanobacterium. Growth of cyanobacterial cultures was assayed by counting cell numbers/mL over a 24 h period. MC-LR (2 μM) did not have any significant effect on cyanobacterial growth, while methyl-viologen (MV), a well-known electron transport blocker inhibited growth in a significant manner (Fig. 5a, a'). This inhibition became significant after 8h of culture (Fig. 5a', a detail of 5a). When MV treatment was combined with MC-LR, growth of *Synechococcus* PCC 6301 was restored compared to MV-only treatments after 2–6 h of treatment (Fig. 5a', arrow), but subsequently declined to levels comparable to MV-only treatments (Fig. 5a, a').

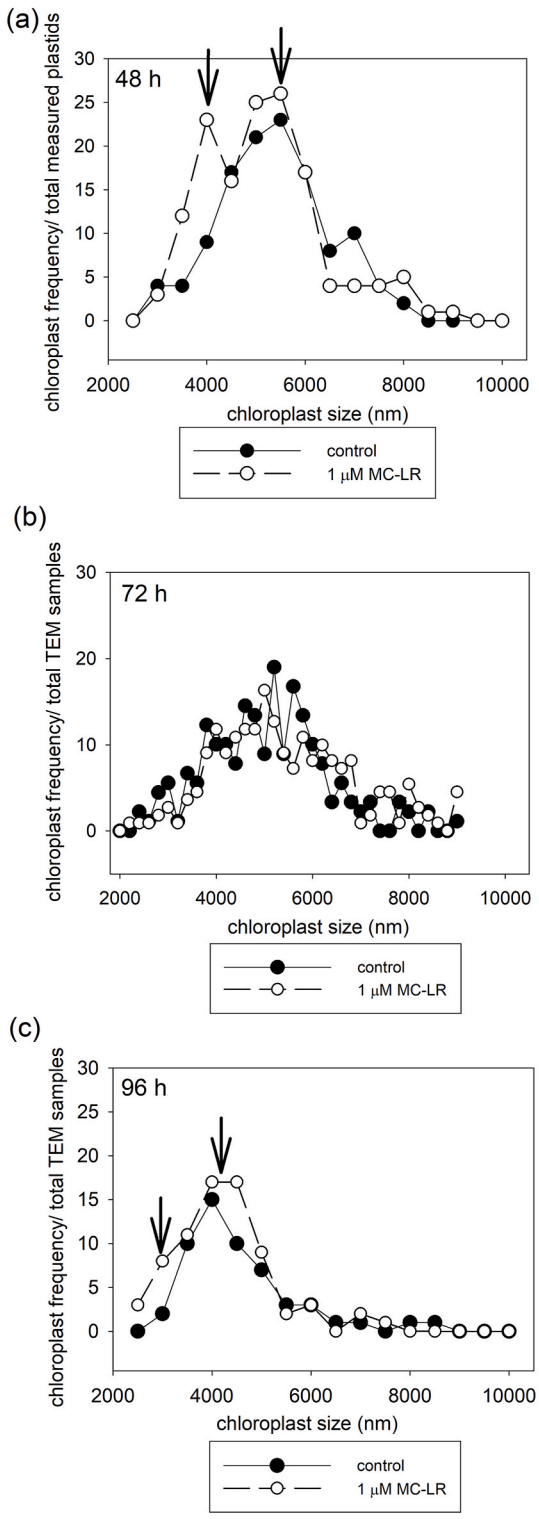

Fig. 3. MC-LR influences chloroplast size distribution in the cortex cells of hypocotyls as revealed by TEM analysis. Exposures to the inhibitor were 48 **(a)**, 72 **(b)** and 96 **(c)** hours. Arrows in (**a, c**) show that MC-LR increases the numbers of small-sized chloroplasts.

Counting both the number of dividing cells mL^{-1} and the percentage of dividing cells out of total cell number showed that MC-LR increased the absolute and relative numbers of dividing/fissioning cells. These increases were significant both at short- and long-term growth of

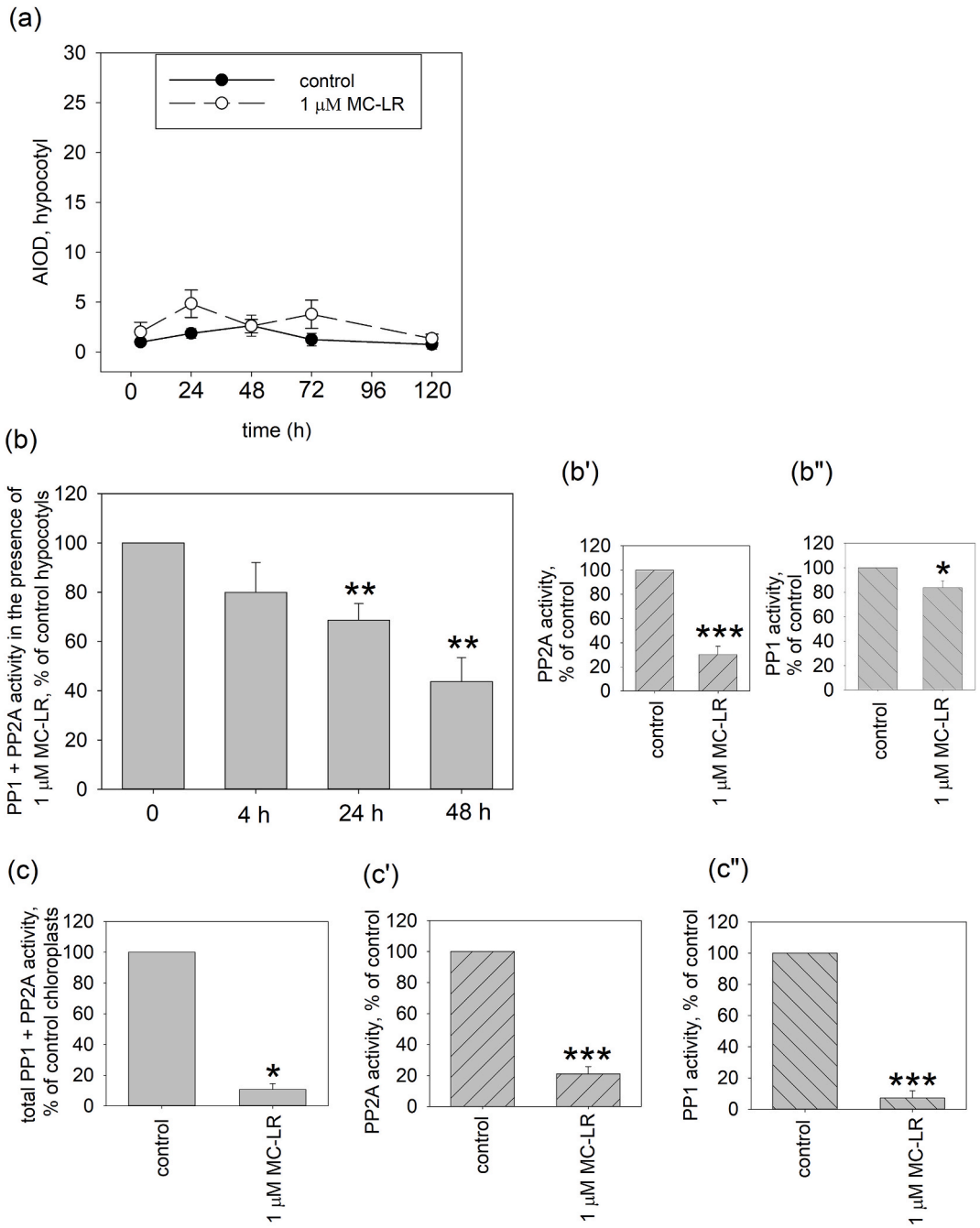

Fig. 4. The effects of MC-LR on ROS formation (a) and on the activities of type 1 and 2A protein phosphatases (PP1 and PP2A) (b, c) in hypocotyls and isolated chloroplasts. **(a)** Time-dependent ROS formation as judged from the intensity of DCFH-DA labeling. AIOD-area integrated optical density, a measure of labeling intensity. **(b)** Time-dependent effects of 1 μM MC-LR on total PP1+PP2A activities. PP2A inhibition **(b')** is more pronounced than PP1 inhibition **(b")** after 48 h of exposure. **(c)** MC-LR alters significantly the total PP1 + PP2A activity in isolated hypocotyl chloroplasts of *Arabidopsis* after 2 h of exposure. Both PP2A **(c')** and PP1 **(c")** activities are severely inhibited. Statistical analysis of differences between controls and treatments was performed by *t*-test and Mann-Whitney Rank Sum tests, *P < 0.05, **P < 0.01, ***P < 0.001.

cyanobacterial cells and contrasted the effects of MV that kept division frequencies at very low levels (Fig. 5b and c). When MV treatments were combined with MC-LR, division/fission was maintained at significantly high levels (compared to MV-only treatments) at most growth times examined (Fig. 5b and c, arrows).

Next, we were conducting comparative analyses of chloroplast division for similar treatments (MC-LR only, MV only, and combined treatments in *Arabidopsis*), although here, MC-LR concentration was reduced to 1 μM, the effective concentration that influenced division/fission. As expected, MC-LR slightly increased and MV decreased division/fission frequency for small chloroplasts and MV decreased the percentage of stage 1 fission significantly (Supplementary Fig. S2/a, arrow). For large chloroplasts, MV did not change the frequency of division stages in total, but decreased stage 1 frequencies in a significant manner (Supplementary Fig. S2/b, arrow). In contrast to what was observed for cyanobacterial cells, the combined MV and MC-LR treatment further reduced division frequencies instead of restoring them. This decrease, which was significant, applied to both small-sized and large chloroplasts (Supplementary Fig. S2/a, b, arrows).

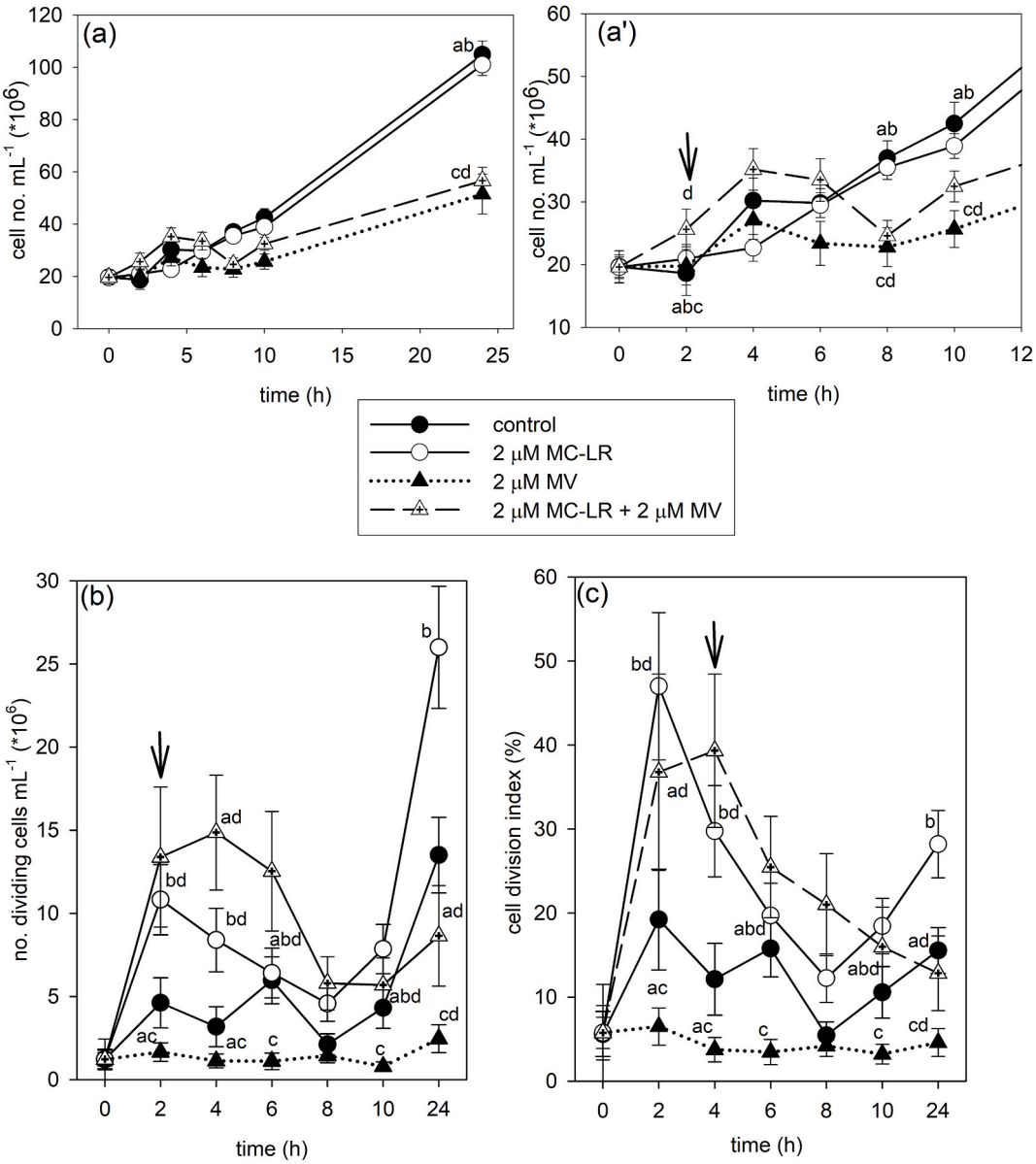

Fig. 5. The effects of MC-LR on the growth and division of *Synechococcus* PCC6301 (a model cyanobacterium) cells exposed to methyl viologen (MV, paraquat) stress. (**a, a'**) The effects of MC-LR, MV and their combinations on cell numbers during growth. (**a'**) is a detail of (**a**) showing growth in the first 10 h after the start of treatments. Arrow shows that if treatment with 2 μM MV is supplemented with 2 μM MC-LR, the growth of culture is maintained in the first 6 h instead of inhibition. **(b, c)** Time-course of the number of dividing cells per mL culture and percentage of dividing cells at treatments as for (a, a'). Arrows on (**b, c**): while MV keeps division at a very low level, MC-LR and the combination of MV + MC-LR increases cell division index in the first 2–6 h of culture. Statistical analysis was performed by Mann-Whitney Rank Sum Test. Lettercodes indicate the significance of differences between the diverse treatments at a given time. Differences between the letter combinations indicate significances. For example, ab vs cd indicate that there are no significant differences within (a–b) and (c–d) respectively, while the ab vs. cd groups show significant differences. a = controls, b = 2 μM MC-LR, c = 2 μM MV, d = 2 μM MC-LR + 2 μM MV.

4. Discussion

Our results show interesting relationships between MC-LR- mediated protein phosphatase inhibition and modulation of chloroplast division. Moreover, the cyanotoxin influences cyanobacterial division as well. Altogether, these changes may have environmental consequences, as discussed below.

Shoots of Arabidopsis seedlings (at least up to 8 days after germination) are characterized by two populations of chloroplasts. Small chloroplasts (longitudinal diameter of ≤6 μm) are predominant in the epidermis and outer cell layers of cortex/mesophyll, while still present in the inner cortex/mesophyll layers. Large chloroplasts (longitudinal diameter of >6 μm) are predominant in the inner cell layers of cortex/mesophyll (Barton et al., 2016, 2017). Their division was stimulated by MC-LR at short-term exposures in the total chloroplast population of hypocotyls. Small-sized chloroplasts, which constitute the larger fraction of the total chloroplast population (Fig. 3) reacted distinctly to cyanotoxin exposure, than large ones. Both short- and long-term exposures increased the percentage of small chloroplasts showing ARC5 signal, while for large ones, only long-term exposures were effective (Figs. 1 and 2). Since chloroplasts in the initial stage of fission did not predominate after cyanotoxin exposure, it can be inferred that the cyanotoxin did not block fission, but rather had a stimulatory effect (Figs. 1 and 2). This may have contributed to the increased number of small chloroplasts per hypocotyl cortex cells as revealed by TEM (Fig. 3). Thus, the cyanotoxin did alter fission activity in a significant manner.

Why TEM analysis did not show any increase in the number of large chloroplasts, while their division was stimulated at long-term MC-LR exposures? We do not have a definite answer to this, but there could be a time delay between the completion of division and the increase in size. According to previous studies, MC-LR induced increased stromule frequency, which was associated to stress reactions. CLSM studies showed that chloroplasts were engulfed in tonoplast-like membrane structures at higher frequencies after MC-LR treatments (Nagy et al., 2018; Juhász et al., 2023). These findings may explain the lack of increase in the number of large chloroplasts. Engulfing of these organelles by tonoplast-like membranes may be followed by functioning of such structures as lytic vacuoles that will degrade these plastids (Nagy et al., 2018).

Clustering of chloroplasts was increased at the ER membranes surrounding the nucleus (Supplementary Fig. S3/a-d and g, h). This phenomenon was observed not only for normally developed plastids, but also for oversized chloroplasts from *arc5* loss-of-function mutants impaired in normal fission (see Pyke, 1997 for a description of the mutant and Schattat et al., 2012 for creating the FNR-GFP containing mutant line) (Supplementary Fig. S3/e, f). Clustering of plastids around the nucleus (through stromules) was shown to be stimulated during plant immune responses to pathogens – HR-PCD (Caplan et al., 2015), although much further work should be performed to reveal the underlying mechanisms. Concerning photosynthetic activity, several studies have reported inhibition by MCs, further underscoring the harmful effects of these cyanotoxins on chloroplast structure and function. These studies included crops, algae, and aquatic macrophytes directly affected by cyanotoxins (see Abe et al., 1996; Saqrane et al., 2009 for examples). Other membrane compartments are also affected by MC-LR in *Arabidopsis*. Vacuolar organization is perturbed and replication/division independent mitochondrial fission seems to be stimulated (Nagy et al., 2018; Juhász et al., 2023). Mutants impaired in the plastid division machinery showed altered photosynthetic activity and stress resistance (Šimková et al., 2012; Dutta et al., 2017). *Arabidopsis* mutants overexpressing PDV, a member of the plastid division machinery, show higher number and smaller chloroplasts as compared to wild-type plants. These genotypes did not show significant functional abnormalities. However, the authors pointed out that optimal number and size of chloroplasts per cell is crucial for maintaining normal metabolic activities (Dutta et al., 2017). Taken together, the evidence presented indicates that MC-LR has hazardous effects on the plastid system and other membrane compartments in plants.

What is the biochemical mechanism for the effects of MC-LR on plastid division? Zheng et al. (2023) raised the hypothesis that MC-LR interacts with FtsZ in the cyanobacterial cell to alter cell division, although they did not provide evidence as to whether this is related to changes in the functionality of prokaryotic protein phosphatases. MC-LR induced alterations of plastid division observed in our study may well be related to its interactions with plastid FtsZ 1/2, but further investigation is needed to confirm this hypothesis. Nonetheless, our study presents some evidence for the biochemical background of these alterations. While DCFH-DA labeling did not show changes in ROS levels in MC-LR treated hypocotyls, we detected cyanotoxin - induced inhibition of both PP2A and PP1 activities both in whole tissues and for the first time, in isolated chloroplasts (Fig. 4). Moreover, without any MC-LR treatment, the fission of small chloroplasts was significantly reduced in seedlings of *c3c4*, an *Arabidopsis* double mutant for catalytic subunits of PP2A (Supplementary Fig. S1; see Spinner et al., 2013 for the description of mutant). Even though other alterations, such as increases in Fe-SOD and Cu/Zn-SOD activities, also occur in seedlings of this mutant (Freytag et al., 2023), the change described above give further proof for the involvement of protein phosphatases in the regulation of chloroplast division. Our findings suggest the possibility of the regulation of plastid division by both cytoplasmic and chloroplast protein phosphatases (see below).

The inhibition of PP2A and PP1-like activities in plastids is interesting from another perspective as well. The existence of such enzymes in plastids is a matter of controversy. An early study stated that there are no such phosphatases in chloroplasts, since protein dephosphorylation activity was not inhibited by MC-LR (Sun and Markwell, 1992). Okadaic acid, a strong inhibitor of PP2A, did alter photosynthetic electron transport in *Dunaliella* plastids, but this was associated to ROS production rather than protein phosphatase inhibition in these organelles (Perreault et al., 2012). However, several types of internal phosphatases, possibly including serine-threonine phosphatases distinct to PP2A/PP1, do exist e.g. to counteract light-dependent phosphorylation of thylakoid proteins, an important mechanism for controlling the functionality of photosystems (Longoni and Goldschmidt-Clermont, 2021). In contrast to previous findings, we did demonstrate the existence of chloroplast phosphatases that can be inhibited by MC-LR in *Arabidopsis*. It is worth mentioning here that knockdown of I-2 protein (an internal inhibitor of PP1) in *Arabidopsis* reduced the levels of phosphoproteins involved in chloroplast development (Ahsan et al., 2017). However, since whole leaf extracts were used in this earlier study, we do not know whether any chloroplast PP1-like enzyme was affected there.

MC-LR affected both chloroplast and cyanobacterial division. While it increased the fraction of dividing *Synechococcus* cells, it did not stimulate cyanobacterial growth (Fig. 5). Our findings are partially different from those of Zheng et al. (2023), a study that showed down-regulation of cyanobacterial division by MC-LR. Our results indicate that the toxin keeps a large fraction of cells in a division stage for longer periods, than in controls, but it does not totally block division activity (it allows growth of the cell population). This effect differs from the modulation of chloroplast division/fission activity (compare Fig. 5 to Figs. 1–3). The observed phenomena raise many questions: does an internal prokaryotic phosphatase regulate cell division of cyanobacteria? Concerning plastids, what are the phosphorylable plastid- and extraplastidial proteins involved? Complex phosphoproteomic studies would help to answer these questions.

As expected, MV treatments inhibited division of small chloroplasts and of *Synechococcus* PCC6301 cells. To our surprise, when MC-LR was used together with MV, it maintained high growth rate and division activity of cyanobacterial cells at early stages of the growth cycle (Fig. 5). Thus, at least for a limited time frame, the cyanotoxin protects cyanobacterial cells from the harmful effects of MV. Based on this observation, we raise the following hypothesis: one of the evolutionary functions of MC-LR is to regulate cell division activity in the producing cyanobacterium and to protect it from (abiotic) stresses. MC-LR is an endotoxin released by cyanobacterial cells only when the producing cell is collapsing (Rastogi et al., 2014). Thus, the competitive advantage of microcystin producers might be related to increased stress resistance of toxin-producing cyanobacterial cells – besides to the harmful effects of MC-LR on other phyto- or zooplankton species. For cyanobacteria, the presence of serine-threonine phosphatases and their involvement in cell division is far from proven, and extensive further research is needed to confirm or refute this relationship. On the other hand, MC-LR treated chloroplasts are not protected from MV in terms of division (Supplementary Fig. S2), so different, partly eukaryotic mechanisms are likely to be involved in the regulation of division here.

According to our results we predict that the phosphoregulation of plastid division is related to both eukaryotic (cytoplasmic) and internal (chloroplast) factors in the plant cell. Our principal arguments supporting this prediction are (i) In parallel to stimulating division of chloroplasts in *Arabidopsis*, MC-LR inhibits PP2A at the whole tissue level and both PP2A and PP1-like activities inside the chloroplasts. Besides, the cyanotoxin influences many processes outside the chloroplasts including mitotic parameters (to be published elsewhere) or the vacuolar system (Nagy et al., 2018). These changes were related to protein phosphatase inhibition, therefore, MC-LR changes the protein phosphorylation pattern outside of plastids as well. Moreover, native extracts of Arabidopsis shoots were subjected to Western blot using anti-MC-LR. Here, organelles were disrupted, and cytosolic/nuclear phosphatases

presumably dominated in these extracts. Blots have shown the binding of MC-LR to a 36 kDa protein - the same or similar molecular weight to PP2A/C and PP1/C (to be published elsewhere). ELISA assays performed to check MC-LR uptake showed that after 24 h of exposure of seedlings to 1 μM MC-LR, shoots contained an average of 4.21 ng g FW^{-1} MC-LR, while roots contained 26.64 ng g FW^{-1}. These cyanotoxin contents were relatively low compared to other plants (Zhang et al., 2021). However, the Western blot results showed strong binding of MC-LR to protein phosphatases inside cells. (ii) MC-LR increases the frequencies of dividing chloroplasts and cyanobacterial cells, but a slight delay of the completion of division can be observed only in cyanobacteria. Further work is needed to identify the molecular targets of this regulatory process. As such, to date, none of the members of the plastid division ring machinery are known to be phosphoregulated. Indirect mechanisms such as perturbation of protein phosphorylation events in photosystems and influencing chloroplast division via such events should be excluded before stating that division is directly influenced by protein phosphorylation.

According to the aims of this work, our principal conclusions are.

(i) MC-LR stimulates the division of chloroplasts (for those of a longitudinal diameter ≤6 μm, not only at long-term but also at short-term) in *Arabidopsis* hypocotyls and this is related to the inhibition of both whole cell/tissue and chloroplast PP2A/PP1 (or, for chloroplasts, at least similar) serine-threonine protein phosphatases. These MC-LR sensitive phosphatases are probably involved in the regulation of plastid division. Since MC-LR does not influence ROS levels in the examined hypocotyls, oxidative stress does not seem to be involved in the observed alterations.
(ii) Interestingly MC-LR influences cyanobacterial division as well, but there is no unequivocal stimulatory effect - the toxin rather induces a slight delay of division exit.
(iii) MC-LR seems to protect cyanobacterial cells from the harmful effects of MV, a strong electron transport blocker. This raises the hypothesis that from an evolutionary perspective, MC production is an ancient mechanism to protect cyanobacterial cells from abiotic stresses. This hypothesis needs further experimental confirmation and does not exclude the other biological functions of the cyanotoxin proposed to date. It would be a future challenge to reveal whether the above effects of MC-LR on cyanobacteria are related to protein phosphorylation events.

Our study has demonstrated for the first time that MC-LR, a cyanobacterial toxin with many harmful effects on aquatic plant communities, affects chloroplast division in a model plant. This is part of a complex network of plant subcellular changes that altogether demonstrate that vascular plants are significantly affected by this cyanotoxin. Further studies are needed to elucidate whether similar changes in plastid division can be observed in MC-LR treated aquatic plant communities or in spray irrigated terrestrial crops.

CRediT authorship contribution statement

Csaba Máthé: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Károly Bóka:** Methodology, Investigation, Formal analysis. **Zoltán Kónya:** Methodology, Investigation. **Ferenc Erdődi:** Methodology, Investigation. **Gábor Vasas:** Methodology. **Csongor Freytag:** Investigation. **Tamás Garda:** Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online athttps://doi.org/10.1016/j.chemosphere.2024.142125

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