



# Microcystin-LR, a cyanobacterial toxin affects root development by changing levels of PIN proteins and auxin response in Arabidopsis roots



Csongor Freytag<sup>a</sup>, Csaba Máthé<sup>a</sup>, Gábor Rigó<sup>b</sup>, Tomasz Nodzyński<sup>c</sup>, Zoltán Kónya<sup>d</sup>, Ferenc Erdődi<sup>d</sup>, Ágnes Cséplő<sup>b</sup>, Erik Pózer<sup>a</sup>, László Szabados<sup>b</sup>, Adrienn Kelemen<sup>a</sup>, Gábor Vasas<sup>a</sup>, Tamás Garda<sup>a,\*</sup>

<sup>a</sup> University of Debrecen, Faculty of Science and Technology, Department of Botany, Egyetem Ter 1., H-4032, Debrecen, Hungary

<sup>b</sup> Biological Research Centre, Institute of Plant Biology, Temesvári Krt 62, H-6726, Szeged, Hungary

<sup>c</sup> Mendel Centre for Plant Genomics and Proteomics, Central European Institute of Technology (CEITEC), Masaryk University, Kamenice 5, 625 00, Brno, Czech Republic

<sup>d</sup> University of Debrecen, Faculty of Medicine, Department of Medical Chemistry, Egyetem Ter 1., H-4032, Debrecen, Hungary

## HIGHLIGHTS

- Microcystin-LR (MCY-LR) is a strongly harmful cyanotoxin for many eukaryotes.
- MCY-LR inhibited protein phosphatase activities in Col-0 Arabidopsis roots.
- In consequence, PIN protein levels were altered within roots, affecting auxin levels.
- MCY-LR altered root development without changing gravitropic bending in Col-0.
- We discuss potential effects of such physiological changes in aquatic ecosystems.

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## ABSTRACT

Microcystin-LR (MCY-LR) is a heptapeptide toxin produced mainly by freshwater cyanobacteria. It strongly inhibits protein phosphatases PP2A and PP1. Functioning of the PIN family of auxin efflux carriers is crucial for plant ontogenesis and their functions depend on their reversible phosphorylation. We aimed to reveal the adverse effects of MCY-LR on PIN and auxin distribution in Arabidopsis roots and its consequences for root development.

Relatively short-term (24 h) MCY-LR treatments decreased the levels of PIN1, PIN2 and PIN7, but not of PIN3 in tips of primary roots. In contrast, levels of PIN1 and PIN2 increased in emergent lateral roots and their levels depended on the type of PIN in lateral root primordia. DR5:GFP reporter activity showed that the cyanotoxin-induced decrease of auxin levels/responses in tips of main roots in parallel to PIN levels. Those alterations did not affect gravitropic response of roots. However, MCY-LR complemented the altered gravitropic response of *crk5-1* mutants, defective in a protein kinase with essential role in the correct membrane localization of PIN2. For MCY-LR treated Col-0 plants, the number of lateral root primordia but not of emergent laterals increased and lateral root primordia showed early development. In conclusion, inhibition of protein phosphatase activities changed PIN and auxin levels, thus altered root development. Previous data on aquatic plants naturally co-occurring with the cyanotoxin showed similar alterations of root development. Thus, our results on the model plant Arabidopsis give a mechanistic explanation of MCY-LR phytotoxicity in aquatic ecosystems.

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## 1. Introduction

Microcystin-LR (MCY-LR) is a natural cyclic heptapeptide toxin synthesized non-ribosomally in several cyanobacterial genera

\* Corresponding author.

E-mail address: [garda.tamas@science.unideb.hu](mailto:garda.tamas@science.unideb.hu) (T. Garda).

(Rastogi et al., 2014). It is known for a long time for its adverse effects on human and animal health as well as its deleterious effects in aquatic ecosystems (Campos and Vasconcelos, 2010). In general, MCY-LR has a double, although probably inter-related effect on eukaryotic cells: (i) it is a potent inhibitor of the serine-threonine protein phosphatases PP1 and PP2A as well as of the minor phosphatases PP4, PP5 and PP6 (Fontanillo and Köhn, 2018; MacKintosh et al., 1990; Máthé et al., 2016). It is known that the inhibitor binds covalently close to the active sites of the catalytic subunits of PP1 and PP2A, causing an irreversible inhibition (see Bouaïcha et al., 2019 for an example). (ii) it is an inducer of reactive oxygen species (ROS) (Máthé et al., 2016, 2019).

The “long” PIN-formed proteins (PIN1–4 and PIN7) are auxin efflux carriers (polar transporters) playing crucial roles in the distribution and functioning of the plant hormone indole-3-acetic acid (IAA, the most abundant auxin): the proper tissue localization of IAA is important for normal growth and development including root tip meristem identity and lateral root formation (Adamowski and Friml, 2015; Barbosa et al., 2018). For roots, PIN1 is transporting IAA from the base towards root tip through the stele, while PIN2 is directing transport from the tip towards the base in the root epidermis and in both directions in root cortex. PIN3 and PIN7 are redistributors of IAA in the root cap columella and are present in root stele as well (Michniewicz et al., 2007a). PIN2, PIN3 and PIN7 are important for gravitropic responses (Adamowski and Friml, 2015; Barbosa et al., 2018; Michniewicz et al., 2007a). Besides PIN1 and PIN2, PIN3 and PIN7 are also involved in lateral root development-proper organization of pericycle cell divisions during initiation of lateral root primordia- and gravitropism (Benková et al., 2003; Michniewicz et al., 2007a; Okumura et al., 2013). Functioning and stability of PINs is regulated by post-translational modifications such as reversible phosphorylation for nearly all PINs and ubiquitination at least for PIN2 (Zwiewka et al., 2019).

The main partners of PIN reversible phosphorylation are PINOID kinase (PID, a serine-threonine kinase) and the serine-threonine protein phosphatase PP2A. PID can phosphorylate all PINs involved in this study (Ganguly et al., 2012; Barbosa et al., 2018). CRK5 involved in this study is a member of the CDPK-related kinase family and it is important in the proper plasma membrane localization of PIN2. The *Arabidopsis* loss-of function mutant *crk5-1* is impaired in root gravitropic bending (Barbosa et al., 2018; Rigó et al., 2013). The reversible phosphorylation of PINs regulates their plasma membrane localization and their dephosphorylation will trigger internalisation and then recycling, usually to the opposite cell side (Kleine-Vehn et al., 2009; Yao and Xue, 2011).

Protein phosphatase inhibitors maintain the phosphorylated state of PIN1 caused by the activity of PID (Michniewicz et al., 2007b). Cantharidin and okadaic acid are strong inhibitors of PP2A activity that alter auxin transport and decrease auxin content in root tips. Cantharidin alters root development (Shin et al., 2005). For MCY-LR, nothing was known to date on its effects on the fate of PINs and consequent auxin distribution. Since it is one of the most abundant naturally occurring cyanotoxins, the relevant studies are necessary. Related to this, in rice roots it decreased auxin levels, inhibiting root elongation and lateral root formation (Chen et al., 2013).

The aim of the present study was to give novel insights into the effects of a natural cyanotoxin on the levels of PIN proteins (PIN 1, 2, 3 and 7) and hence, of auxin (IAA) within *Arabidopsis* roots and the consequent alterations in root development and gravitropism. We discuss the possible environmental implications of these alterations.

## 2. Materials and methods

### 2.1. The purification of microcystin-LR

MCY-LR was purified from *Microcystis aeruginosa* BGSD 243 according to Kós et al. (1995) modified by Vasas et al. (2004). Cells were collected by centrifugation, then extracted as described. Purification of cyanotoxin was performed on a DEAE Cellulose-52 (Whatman DE 52) column. The eluates containing MCY-LR were further purified by Toyopearl Size Exclusion Chromatography. The purity of the preparations was verified by HPLC (C-18 HPLC column, Supelcosil TM SPLC-18, 25 cm 10 mm, 5 mm). HPLC analyses were performed with a Shimadzu HPLC system equipped with a Shimadzu SPD-M 10 AVP diode array. The distinctive peaks of the chromatogram were checked by LC-MS. MCY-LR was detected as  $[M+H]^+$  at  $m/z$  995.5.

### 2.2. Plant material and MCY-LR treatments

The *Arabidopsis thaliana* genotypes used in this study were: Col-0 (wild-type), Col-0 plants bearing PIN1:GFP, PIN2:GFP, PIN3:GFP (PIN1:PIN1-GFP, PIN2:PIN2-GFP and PIN3:PIN3-GFP) (Benková et al., 2003; Xu et al., 2006; Žádníková et al., 2010), DR5rev:GFP fusion constructs for the detection of tissue areas characterized by the presence of and responses to auxins (Friml et al., 2003b; Žádníková et al., 2010) and the *crk5-1* loss of function mutant (Rigó et al., 2013).

Seeds were surface sterilized by rinsing two times for 10 min with 10% of a sodium-hypochlorite containing solution, then washed five times for 5 min with sterile water. Seeds were transferred to MS (Murashige and Skoog, 1962) basal medium supplemented with Gamborg's vitamins, 2% (w/v) sucrose (Molar, Budapest, Hungary) and 0.8% (w/v) Bacto-agar (Difco, Lawrence, KS, USA) (Gamborg et al., 1968; Murashige and Skoog, 1962). After a 48-h cold treatment, plates were placed in a tissue culture room (14/10 h photoperiod,  $22 \pm 2$  °C,  $60 \mu\text{mol m}^{-2}\text{s}^{-1}$  photon flux density in the light period). After 5 days of germination, seedlings were treated with MCY-LR under the above specified physical laboratory conditions (see Nagy et al. (2018) for the method of treatments). The study of gravitropic responses was performed with 3 days old seedlings.

### 2.3. Immunohistochemistry and histochemistry methods. Microscopy

Seedlings bearing the GFP-fusion constructs (PIN:GFP and DR5rev:GFP) were examined with an Olympus FluoView 1000 confocal microscope (excitation wavelength: 488 nm, emission wavelength range: 500–530 nm;  $\times 60$  water-immersion UPLSAPO objective, with a numerical aperture of 1.2, acquisition software: FV-1000). For the analysis of signal intensity, we constructed 2D projection images, then 2D heatmaps of these images were constructed for a better visualization. Mean grey values (AIODs, Area Integrated Optical Densities) were calculated with the aid of Fiji (ImageJ-Win64) software after background subtraction of 2D heatmap images. For GFP signals in DR5rev:GFP plants, heatmaps from 3D images of confocal microscopic stacks for tips and differentiated segments of main roots were also constructed. “Differentiated root segments” or “-tissues” means the maturation zone of main (primary) root throughout this study. 3D Heatmaps were obtained with the aid of ZEN Blue 2.3 Lite software and showed local intensities and areas of GFP signals in root segments.

The whole-mount immunolocalization of PIN1, PIN2 and PIN7 was performed by the use of InsituPro VSi pipetting robot as described previously (Friml et al., 2003a; Sauer et al., 2006). We fixed Col-0 main root tips with 4% (w/v) paraformaldehyde (PFA) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>•2H<sub>2</sub>O, 0.5 mM MgCl<sub>2</sub>•6H<sub>2</sub>O/pH 7.4 adjusted with HCl) for 40 min under vacuum. Samples were then washed with PBS/0.1% Triton-X100 (3 × 15 min) and deionized water/0.1% Triton-X100 (3 × 15 min). Cell walls were digested with 2% Driselase (Sigma-Aldrich, St. Louis, Mo., USA) in PBS for 30 min at 37 °C and samples were washed with PBS/0.1% Triton-X100 (3 × 15 min). In the next step the seedlings were permeabilized with 10% DMSO/3% IGEPAL in PBS for 30 min and washed with PBS/0.1% Triton-X100 (3 × 15 min). Samples were pre-incubated in 2% BSA/PBS for 1 h and incubated with primary antibody in 2% BSA/PBS for 4 h at 37 °C, then washed with PBS/0.1% Triton-X100 (5 × 15 min). Rabbit raised anti-PIN1, anti-PIN2 (Skokan et al., 2019) and anti-PIN7 (Doyle et al., 2019) were used as primary antibodies. Secondary antibody was Cy3 conjugated anti-rabbit IgG generated in sheep (Sigma-Aldrich, C2306). Incubation with secondary antibody was in 2% BSA/PBS for 4 h at 37 °C, then samples were washed with PBS/0.1% Triton-X100 (3 × 15 min) and deionized water (3 × 15 min). Preparations were analyzed with a Zeiss LSM Z700 confocal microscope with the conventional settings for Cy3 visualization. Data evaluation was performed by comparing the signal intensity of control plants with signal intensity of treated ones. In all cases, we worked with the same microscope settings that included laser intensity, gain, objective and magnification.

Reactive oxygen species (ROS) were detected in Col-0 roots according to Garda et al. (2016). We have used the fluorescent dye 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich) at a concentration of 20 μM. The dye is excitable at 450–480 nm. We used an Olympus Provis AX-70 fluorescence microscope (Olympus, Tokyo, Japan) for observations and labelling intensities were quantified with ImageJ 64 software.

In all experiments described in this subchapter, at least five seedlings per treatment per experiment were used and four-five independent experiments were performed.

#### 2.4. Investigation of root development and gravitropic responses

Gravitropic response assay was performed essentially as described by Rigó et al. (2013) with modifications. Three days-old Arabidopsis Col-0 and *crk5-1* mutant seedlings were used for treatments. For the study of gravitropic bending, seedlings were grown vertically on half-strength MS medium with 1% (w/v) sucrose, in contrast to Rigó et al. (2013), where reduced sucrose concentration (0.5%) was used. Afterwards, seedlings were transferred to the same culture medium for control and 1 μM MCY-LR treatments. The vertically grown seedlings were reoriented by 135°, and the degree of root bending was recorded by scanning 24 h after rotation. The rate of root bending was determined by measuring the angle formed between the horizontal baseline and growth direction of root tip as described—that is, the angle between reoriented starting position and a 24-h position (Rigó et al., 2013). At least 100 wild-type and *crk5-1* seedlings were tested per each treatment in three separate experiments.

For the study of development of lateral root primordia and emergent laterals, non-gravistimulated five days old Col-0 seedlings were MCY-LR treated for one and two days. PFA-fixed roots were labeled for chromatin with DAPI (see above, Immunohistochemistry and histochemistry), that showed clearly not only emergent laterals, but primordia as well. We considered as primordia all stages of lateral root development before their

emergence from the main (primary) root. Whole roots were scanned by microscopy for lateral root numbers. The distance between tips of main roots and the occurrence of the first lateral root primordium was also measured. At least 200 seedlings were tested per each treatment and experiments were repeated four times.

#### 2.5. The assay of protein phosphatase activities

Total protein phosphatase activity, which includes PP1 and PP2A activities *in vivo*, was measured as described previously (Garda et al., 2018; Máthé et al., 2013). Arabidopsis Col-0 whole roots were extracted with a buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 mM EGTA, 0.1% (w/v) β-Mercaptoethanol, 1 mM PMSF (Sigma-Aldrich), 0.5% (v/v) protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA). The protein content of extracts was determined according to Bradford (1976) so that the assays were carried out with the same amounts of protein. <sup>32</sup>P-MLC20 (phosphorylated turkey gizzard 20 kDa myosin light chain labeled with <sup>32</sup>P) was used as substrate. Specific phosphatase activities were given as pmol [<sup>32</sup>Pi] released mg protein<sup>-1</sup> (Erdődi et al., 1995) and presented as the percentage of control activities (100%). Protein phosphatase activity assays were performed in three parallel measurements per treatment and three separate experimental replicates.

#### 2.6. Data analysis

All quantified data were plotted —plots are showing the mean ± SE values—with the aid of Systat Sigma Plot 10.0 ® software except for data on gravitropic responses plotted with R studio version 1.2 with ggplot2 graphical package. Statistical significances for the differences between controls and treatments were studied by Mann-Whitney Rank Sum Test and t-tests. Differences were considered to be significant at P < 0.05.

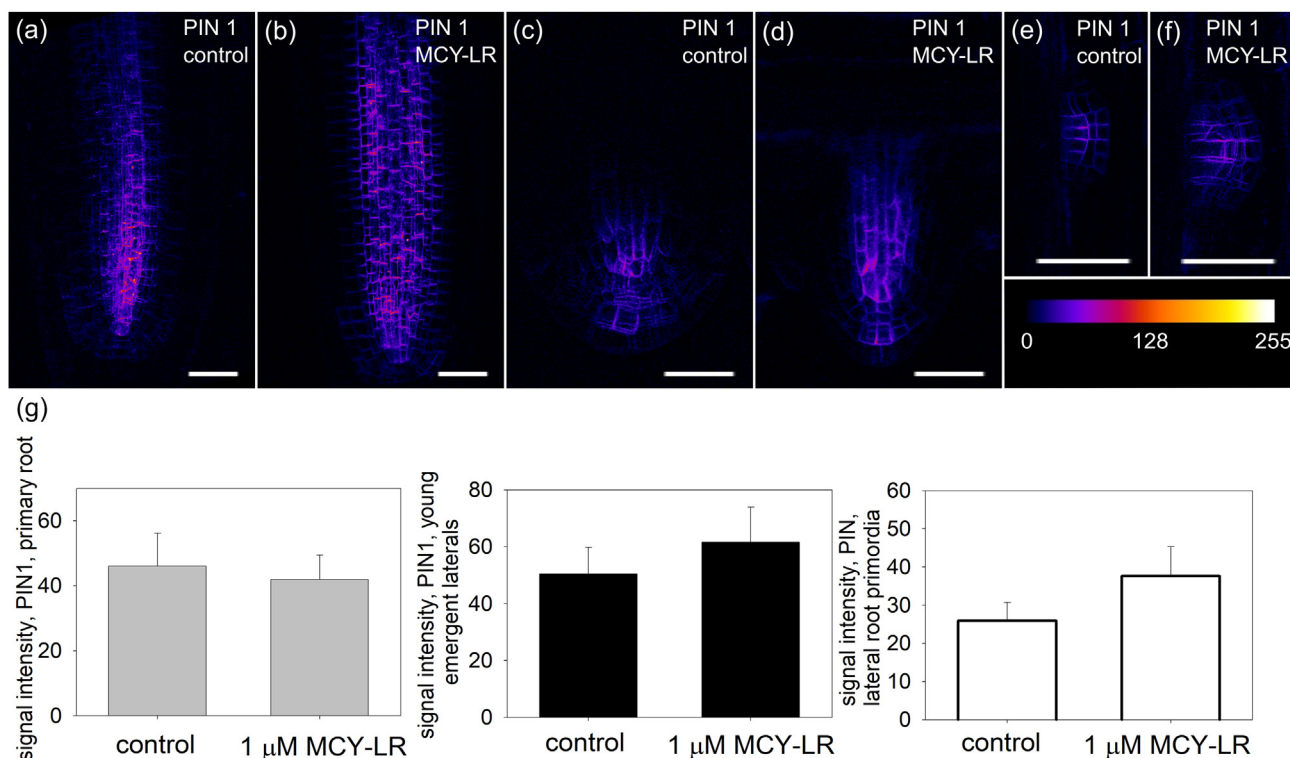
### 3. Results

Our preliminary studies employed the use of MCY-LR in a concentration range of 1–10 μM. Concentrations of 5 μM and above induced cell lethality at long and even short term exposures, so they were omitted (data not shown). 1 μM MCY-LR induced sufficient protein phosphatase inhibition after 24 h of exposure without inducing rapid cell death, therefore this treatment was used throughout our experiments. The above findings confirm the “dualistic response” hypothesis for the effects of microcystins in plants (Corbel et al., 2015). The relatively short term treatments with 1 μM MCY-LR allowed us to get insights into the PIN/auxin content-root development relationship.

#### 3.1. Effects of MCY-LR on the levels of auxin efflux carriers (PIN1, PIN2, PIN3, PIN7)

PINs were analyzed in PIN:GFP plants and by immunohistochemistry. Both approaches showed their normal polar localization and distribution at the subcellular and tissue level in roots of control *Arabidopsis thaliana* seedlings. For primary roots, PIN1 protein was normally localized in the stele and PIN2 protein in the cortex and epidermis (Figs. 1a and 2a, Supplementary Fig. S1/a, c, e). Immunohistochemical labeling showed clear decreases of PIN levels after MCY-LR treatments at each test period. 1 μM MCY-LR reduced the level of PIN1 and PIN2 proteins after one (Supplementary Fig. S1/b), two (Supplementary Fig. S1/d) and five (Supplementary Fig. S1/f) days as compared to control plants. For PIN:GFP plants, MCY-LR decreased PIN1 and PIN2 levels after one





**Fig. 1.** The effects of MCY-LR on PIN1 content of primary and lateral roots. (a–f) Intensities of color-coded (heat-mapped) 2D maximum projections of CLSM images of PIN1:GFP signals from tips of non-gravistimulated roots show that 24 h treatment with 1  $\mu$ M MCY-LR modifies PIN levels in Arabidopsis. (a, b)– primary roots; (c, d)– emergent laterals; (e, f)– lateral root primordia. Heatmap scale is shown. Size scalebars: 50  $\mu$ m. (g) Quantification of PIN1:GFP signals from Arabidopsis root tips show differential effects of MCY-LR on PIN levels in primary vs. lateral roots at 24 h treatments. Signal intensity was calculated as AIOD (mean grey value, area integrated optical density/AIOD). Left: primary roots, centre: emergent lateral roots, right: lateral root primordia. See Section 2.3. of Materials and methods for sample numbers and Section 2.6. for the methods of statistical analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

day treatment as well (Figs. 1b and 2b). However, these decreases were non-significant (Figs. 1g and 2e/primary roots).

In contrast to primary roots, GFP signals for both PIN1 and PIN2 increased in emergent laterals in the presence of MCY-LR (Figs. 1d and 2d) and this increase was significant for PIN2:GFP (Fig. 2e/emergent laterals). For lateral root primordia, 1  $\mu$ M MCY-LR induced a slight, but non-significant increase of PIN1:GFP signal (Fig. 1 e, f, g/lateral primordia). For PIN2:GFP, there was a minimal number of lateral root primordia in the young seedlings investigated, so we could not quantify this parameter. Data for lateral roots refer only to 24 h treatments with the cyanotoxin.

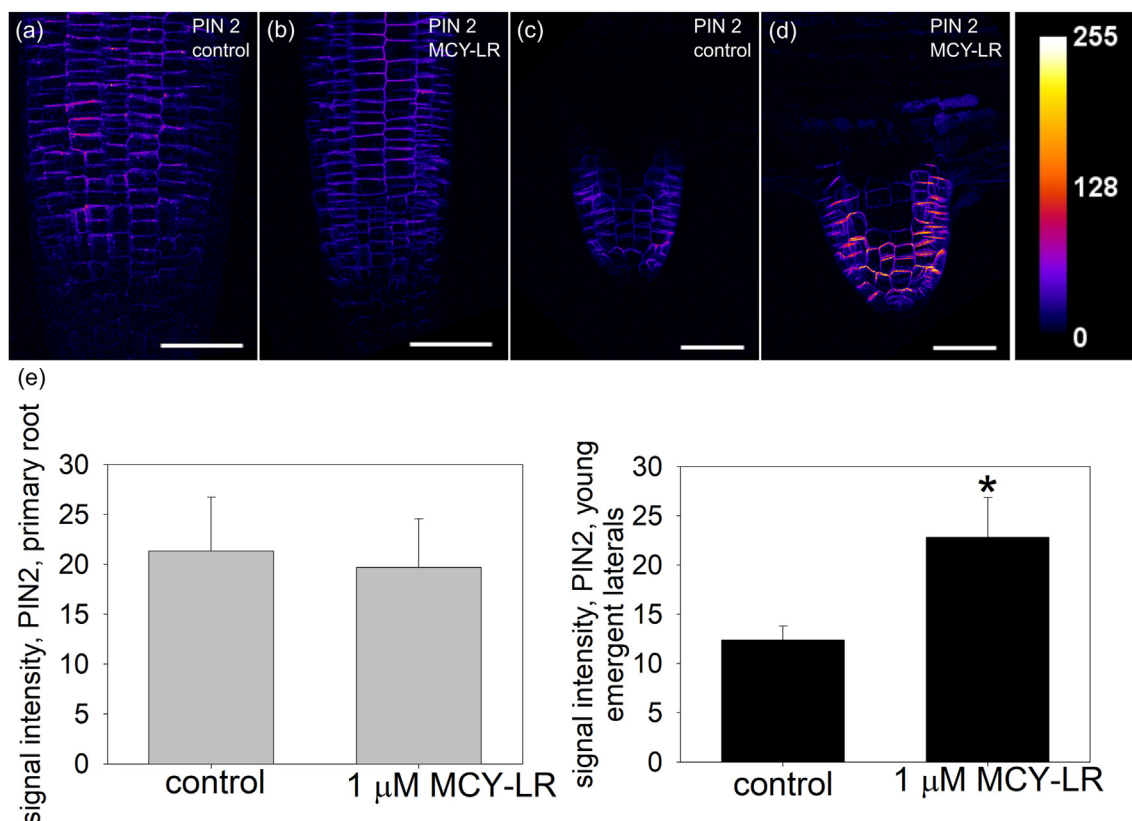
For PIN3:GFP plants, untreated primary roots showed strong GFP expression in root cap columella and weaker expression in stele (Fig. 3a). At relatively short term (24 h) MCY-LR exposures, GFP signals increased both in the columella and stele of primary root tips and for the stele, this change was significant (Fig. 3b, e/primary root columella and stele). There was no sufficient number of PIN3:GFP emergent laterals to perform quantification. For lateral root primordia, MCY-LR had decreased PIN3:GFP signal in a significant manner (Fig. 3c, d, e/lateral root primordia). After seven days of exposure, 1  $\mu$ M MCY-LR caused a decrease of PIN3:GFP signal in primary roots (data not shown).

Immunohistochemical labeling of PIN7 in primary roots showed its normal distribution with high levels in the stele and distal segments of root apex in control plants (Supplementary Fig. S2/a, a', c, e, g). At 1  $\mu$ M MCY-LR treatments, the level of PIN7 was reduced in the stele at each test time (Supplementary Fig. S2/b, d, f, h) and distal segments of root apex after 24 h of exposure (Supplementary Fig. S2/b').

### 3.2. Effects of MCY-LR on auxin levels in roots

The levels of and responses to auxin in roots were analyzed by the examination of GFP signals in *A. thaliana* plants bearing the auxin responsive DR5rev:GFP construct. Quantification of 2D heat map projections of GFP signals showed non-significant, but consistent decreases of auxin levels/responses in tips of primary roots (Fig. 4 a, b, e). To get more precise data on distribution of auxin content/response in root tissues, 3D heat map projections of GFP signals were constructed. These showed the distribution of auxin in the apex and differentiated segments of primary roots. For control plants, GFP signals were detected mostly in root cap columella and stele of root tips (Supplementary Fig. S3/a, e) and in the subepidermal cell layers and stele of differentiated root segments (Supplementary Fig. S3/c, g). 24 h of treatment with 1  $\mu$ M MCY-LR induced decreases of intensities and areas of GFP signals within root tips (Supplementary Fig. S3/b) and differentiated root segments (Supplementary Fig. S3/d). For the latter, auxin levels/responses decreased well visibly in stele (Supplementary Fig. S3/d, arrowhead). For 48 h treatments with 1  $\mu$ M MCY-LR, auxin levels were reduced as well (Supplementary Fig. S3/f, h).

The 24-h treatment with 1  $\mu$ M MCY-LR resulted in a well visible decrease of GFP signal in emergent lateral roots of DR5rev:GFP plants (Fig. 4c and d). However, there were very few emergent lateral roots or lateral root primordia in the young DR5rev:GFP seedlings, thus we could not perform a thorough quantification of results here.



**Fig. 2.** The effects of MCY-LR on PIN2 content of primary and lateral roots. (a–d) Intensities of color-coded (heat-mapped) 2D maximum projections of CLSM images of PIN2:GFP signals from tips of non-gravistimulated roots show that 24 h treatment with 1  $\mu$ M MCY-LR modifies PIN2 levels in Arabidopsis. (a, b)– primary roots; (c, d)– emergent laterals. Heatmap scale is shown. Size scalebars: 50  $\mu$ m. (e) Quantification of PIN2:GFP signals from Arabidopsis root tips show differential effects of MCY-LR on PIN levels in primary vs. lateral roots at 24 h treatments. Signal intensity was calculated as AIOD (mean grey value, area integrated optical density/AIOD). Left: primary roots, right: emergent lateral roots. Asterisk shows a significant difference of MCY-LR treatments vs. controls. See Section 2.3. of Materials and methods for sample numbers and Section 2.6. for the methods of statistical analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 3.3. Effects of MCY-LR on root gravitropic response and development

As the experiments with DR5rev:GFP plants revealed that MCY-LR interferes with auxin levels/responses, we became interested how this inhibitor can influence auxin-controlled responses such as root gravitropism or root development. MCY-LR did not affect the gravitropic response of Col-0 plants (Fig. 5a). Gravitropic bending was inhibited in the control *crk5-1* loss-of function mutant (see Introduction for the function of CRK5) (Fig. 5a): the percentage of plants showing maximum 90° bending increased and the percentage of plants restoring 135° bending decreased (Fig. 5a). These observations confirmed those of Rigó et al. (2013), even under different physical laboratory conditions and increased sucrose content of culture medium. Interestingly, the abnormal gravitropic response of *crk5-1* mutant was restored to a level close to control by MCY-LR (Fig. 5a).

For non-gravistimulated Col-0 plants, the effects of 1  $\mu$ M MCY-LR on the number of lateral root primordia and emergent lateral roots were time -dependent (Fig. 5b and c). MCY-LR treatment promoted lateral root initiation, since it increased significantly the number of primordia after two days of exposure. At this stage the number of primordia was nearly doubled as compared to the start of experiment, while in case of controls, development of primordia was relatively slow (Fig. 5b). For emergent laterals, their number decreased, but in a non-significant manner even after two days of exposure (Fig. 5c). This meant that development of lateral root

primordia was stimulated by the cyanotoxin, but this was not followed by the promotion of their further development.

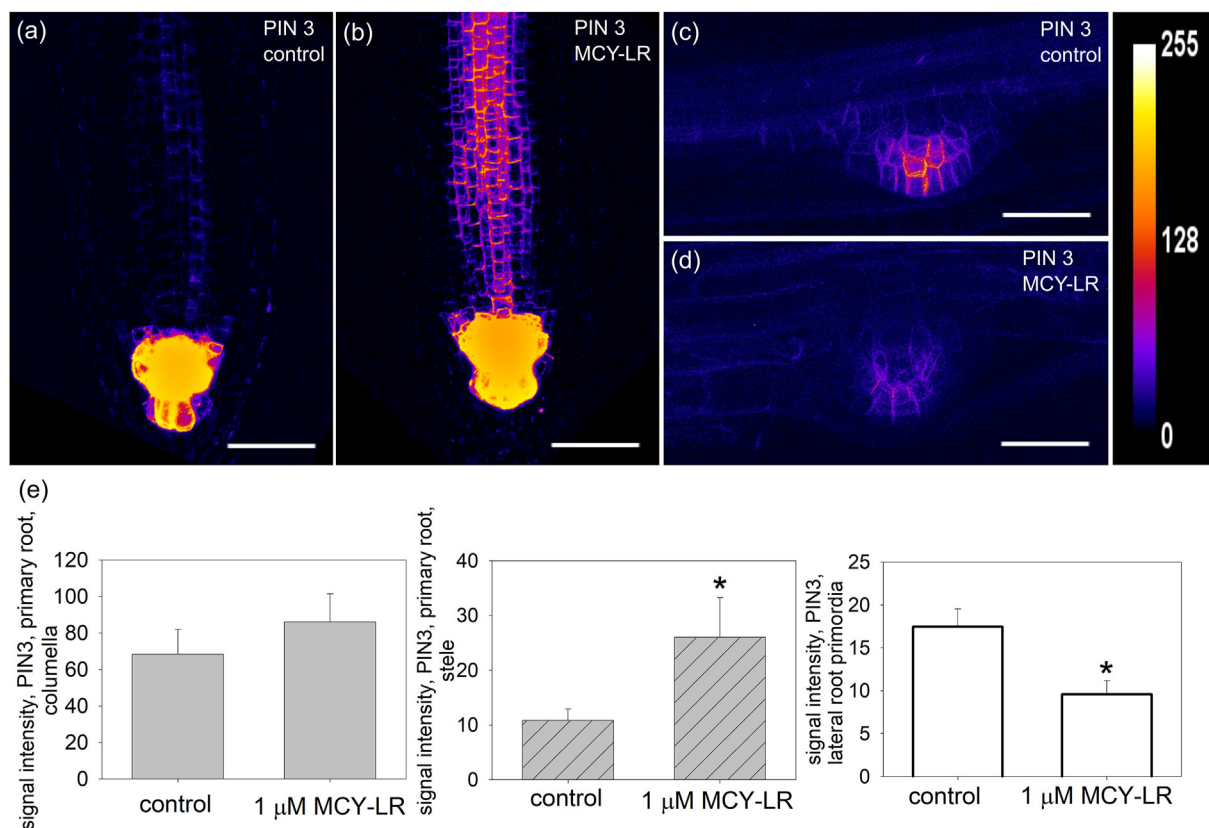
MCY-LR inhibited the elongation growth of main roots in Col-0 plants. 1  $\mu$ M MCY-LR reduced significantly the distance of the first lateral root primordium from the main root tip after 24 h treatments. This effect persisted after two days of treatments (Supplementary Fig. S4).

### 3.4. The effects of MCY-LR on ROS production and protein phosphatase activities in Col-0 roots

In Col-0 seedlings, MCY-LR did not alter ROS levels in primary root tips at 24 h treatments (Fig. 6a). Only a long-term (5 days) treatment with the cyanotoxin induced a significant increase of ROS levels (data not shown). In differentiated root segments ROS levels did not change significantly for up to three days of MCY-LR treatments (Fig. 6b), while they were decreased significantly after seven days of exposures with the phosphatase inhibitor (data not shown). The total serine-threonine protein phosphatase (PP1 and PP2A) activities were significantly inhibited (nearly 50% inhibition) by 1  $\mu$ M MCY-LR in whole root extracts of plants treated for 24 h (Fig. 6c).

## 4. Discussion

Several harmful effects of MCY-LR on plant cells/tissues are known. This includes alterations of cytoskeleton and chromatin



**Fig. 3.** The effects of MCY-LR on PIN3 content of primary and lateral roots. (a–d) Intensities of color-coded (heat-mapped) 2D maximum projections of CLSM images of PIN3:GFP signals from tips of non-gravistimulated roots show that 24 h treatment with 1  $\mu$ M MCY-LR modifies PIN3 levels in Arabidopsis. (a, b)– primary roots; (c, d)– lateral root primordia. Heatmap scale is shown. Size scalebars: 50  $\mu$ m. (e) Quantification of PIN3:GFP signals from Arabidopsis root tips show differential effects of MCY-LR on PIN levels in primary vs. lateral roots at 24 h treatments. Signal intensity was calculated as AIOD (mean grey value, area integrated optical density/AIOD). Left: primary roots, columnella, centre: primary roots, stele, right: lateral root primordia. Asterisks show significant differences of MCY-LR treatments vs. controls. See Section 2.3. of Materials and methods for sample numbers and Section 2.6. for the methods of statistical analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

organization, cell cycle regulation, cell death/tissue necrosis (Jámbrik et al., 2011; Máthé et al., 2013, 2016; Pappas et al., 2020).

Auxin as a plant growth regulator is crucial for root development in all vascular plants. Any perturbation of its local concentration will alter root and shoot development. Its distribution within the plant body depends largely on the proper functioning of PIN efflux carriers (Adamowski and Friml, 2015). Functioning of PINs is regulated by reversible phosphorylation (Friml et al., 2004; Zwiewka et al., 2019). This paper shows how local amounts of PINs and in consequence auxin are altered in roots of Arabidopsis in the presence of MCY-LR, a potent protein phosphatase inhibitor. *rcn1*, *pp2aa1,2*, mutants lacking a functional “A” regulatory subunit of PP2A show alterations of primary and lateral root development as well as gravitropic response of roots (Michniewicz et al., 2007b; Rashotte et al., 2001; Zhou et al., 2004) and as we will see, MCY-LR has several effects similar to this mutant phenotype.

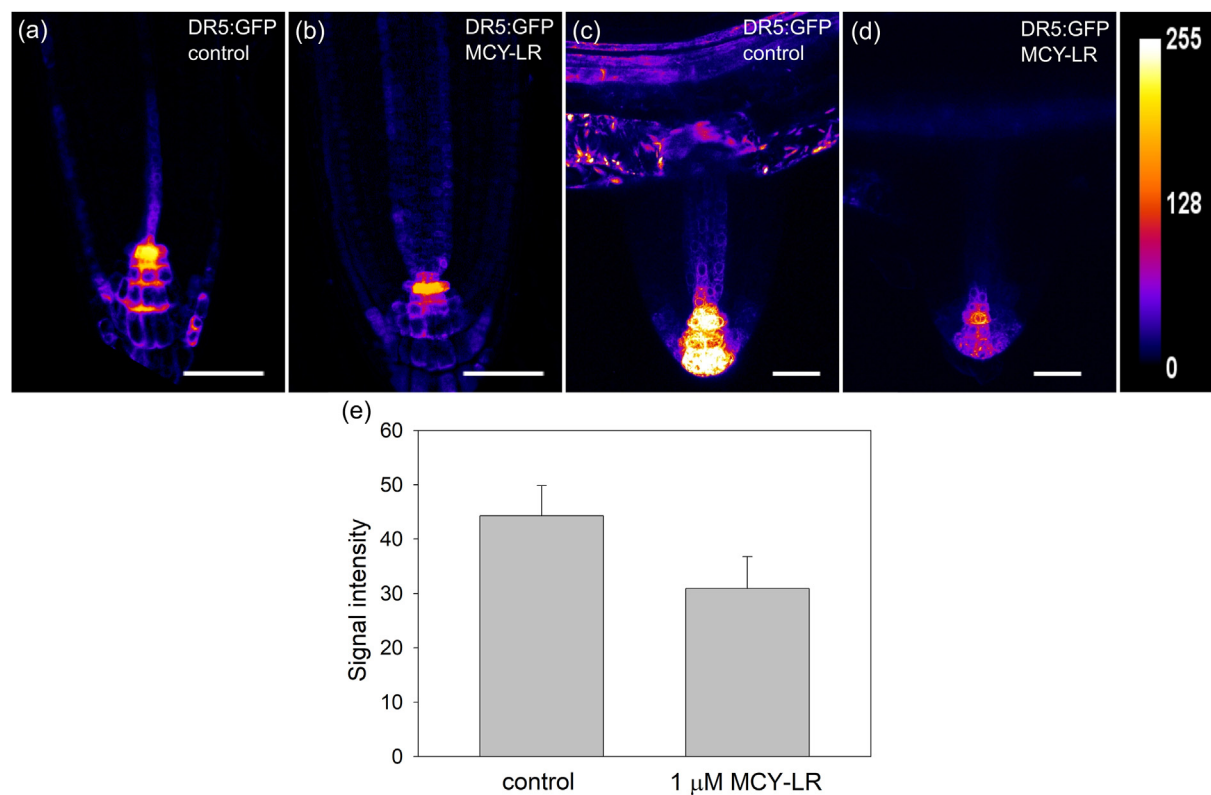
The levels of PIN1, PIN2 and PIN7 decrease in the tips of Arabidopsis primary roots treated with MCY-LR. Plants bearing PIN:GFP constructs show a non-significant decrease, while the immunohistochemistry approach shows a more dramatic change (Figs. 1–2 and Supplementary Figs. S1–S2). Interestingly cantharidin, another natural potent inhibitor of PP2A (MacKintosh and Diplexico, 2009) did not alter PIN2 levels in root tips (Shin et al., 2005). For PIN3, MCY-LR has opposing effects since its level increases in primary root tips (Fig. 3). For lateral roots, the cyanotoxin has contrasting effects-levels of PIN1 and PIN2 increase and those of PIN3 (in lateral root primordia) decrease here (Figs. 1–3). This suggests that MCY-

LR induces redistribution of PINs in roots. The biochemical background is the inhibition of protein phosphatase activity, while ROS levels do not change at short-term exposure to the cyanotoxin (Fig. 6). Thus, the potential side-effects of the cyanotoxin like ROS induction can be excluded during 24-h treatments and the changes in PIN/auxin levels can be directly related to the specific effects of MCY-LR on protein phosphatases. Changes of auxin levels/responses are expected after such changes in the localization of PINs and indeed, plants bearing DR5rev:GFP constructs showed non-significant, but consistent decrease of auxin levels in tips of primary roots (Fig. 4 and Supplementary Fig. S3).

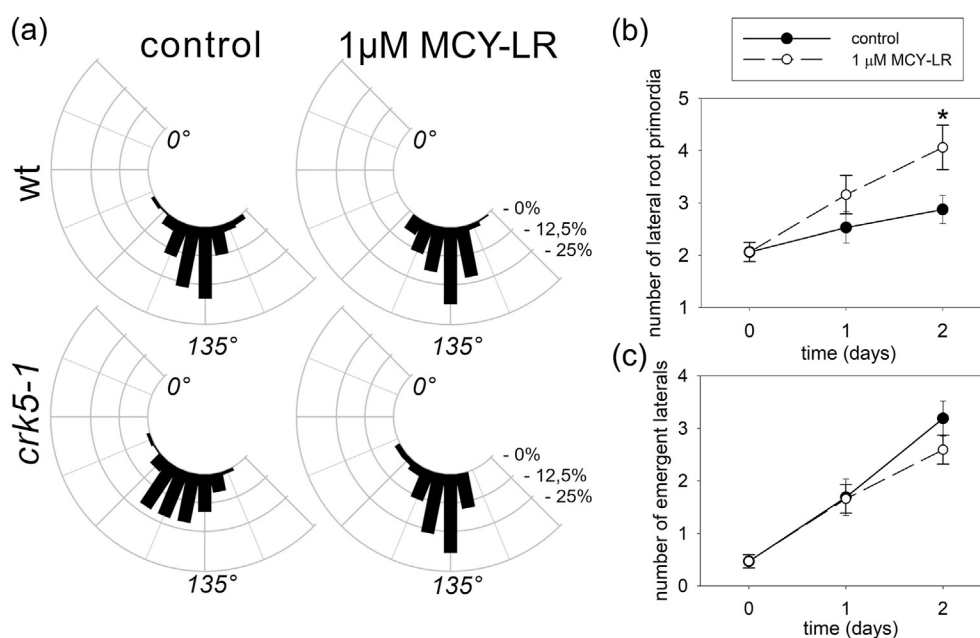
What is the consequence of these changes for root development? Again, the Arabidopsis mutants for PP2A scaffolding, regulatory or catalytic subunits show that perturbation of PP2A activity or its subcellular/tissue localization inhibits longitudinal growth of primary roots, stimulates the formation of lateral root primordia but inhibit their elongation. This is partly a consequence of auxin mislocalization (Zhou et al., 2004; Michniewicz et al., 2007b; Spinner et al., 2013). We observed very similar root developmental alterations after MCY-LR treatments. The distance between root tip and the first lateral root primordium decreased, which means the elongation of primary root was inhibited (Supplementary Fig. S4). Meanwhile, development of lateral root primordia was stimulated and this was not followed by stimulation of lateral root emergence (Fig. 5 b, c).

PIN2, PIN3 and PIN7 are important for the gravitropic response of roots and decrease of their levels inhibits gravitropic bending

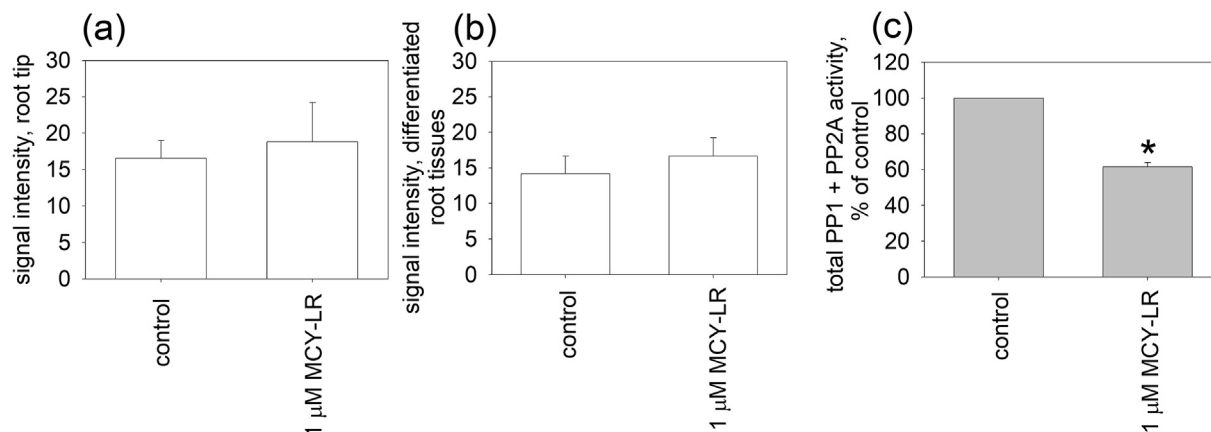




**Fig. 4.** (a–d) Heat-mapped 2D maximum projections of CLSM images of DR5rev:GFP signals from tips of non-gravistimulated primary and lateral roots show that 1  $\mu$ M MCY-LR decreases auxin levels in Arabidopsis after 24 h of treatment. (a, b) - primary roots; (c, d) emergent laterals. Heatmap scale is shown. Size scalebars: 50  $\mu$ m. (e) quantification of DR5rev:GFP signals from Arabidopsis root tips show decreases in the presence of MCY-LR. Signal intensity was calculated as AIOD (mean grey value, area integrated optical density/AIOD). See Section 2.3. of Materials and methods for sample numbers and Section 2.6. for the methods of statistical analysis.



**Fig. 5.** (a) MCY-LR restores the abnormal gravitropic response of *crk5-1* mutants. Plants were gravistimulated as described by Rigó et al. (2013). This Figure represents bending of primary roots after the 135° reorientation (see Methods). Lower bending degrees than 135° indicate abnormal gravitropic response. Complete root (re)bending is of 135° (arrows). (b, c) - Time- and dose dependent effects of MCY-LR on lateral root development in non-gravistimulated roots of Col-0 seedlings: (b) number of lateral root primordia; (c) number of emergent laterals. Asterisk on (b) indicates significant difference between controls and treatment. See Section 2.4. of Materials and methods for sample numbers and Section 2.6. for the methods of statistical analysis.



**Fig. 6.** (a, b) The effects of 24 h MCY-LR treatment on ROS levels in root tips (a) and differentiated root tissues (b) visualized by the intensity of DCFH-DA staining. Signal intensity was calculated as AIOD (mean grey value, area integrated optical density/AIOD). (c) The effect of 1  $\mu$ M MCY-LR on total protein phosphatase activity at 24 h of exposure. Asterisk indicates significant difference of controls vs. treatments. See Section 2.3. of Materials and methods for sample numbers and Section 2.6. for the methods of statistical analysis.

(Adamowski and Friml, 2015; Michniewicz et al., 2007a). MCY-LR did not alter this response, probably because increase of PIN3 levels in primary root tips compensated for the decreases of PIN2 and PIN7. Interestingly, the cyanotoxin restored gravitropic response of *crk5* mutants (Fig. 5a). This mutant lacks a CDPK type protein kinase that contributes to PIN2 phosphorylation (Rigó et al., 2013). As a consequence, PIN2 levels in the transition zone of vertically grown roots decrease and the shift of PIN2 to asymmetric distribution during gravistimulation is delayed. This will result in the perturbation of gravitropic response in mutants (Rigó et al., 2013). The effect of MCY-LR may be caused by maintenance of PIN2 phosphorylated state by an alternative, yet unknown kinase and parallel inhibition of PIN2 dephosphorylation by a PP2A complex. This is an exciting issue that needs further investigation. In contrast to MCY-LR, cantharidin reduces gravitropic bending of roots, similarly to the Arabidopsis *rcn1* mutant, defective in the A regulatory subunit of PP2A (Rashotte et al., 2001). Other loss of function mutants of the *pp2aa* series – again, affecting A subunits – are also inducing altered gravitropism- and the collapse of root apical meristem (Xi et al., 2016). What can be the reason for the differential effects of cantharidin? The studies on cantharidin (otherwise known to be potent inhibitor of PP2A) mentioned in this paper did not include protein phosphatase activity assays and we can suppose it had differential effects to MCY-LR presented here in this respect.

What can be the environmental consequence of such alterations in PIN/auxin distribution? MCY-LR induces serious impairment in the development of primary and lateral roots of *Phragmites australis*, an aquatic plant naturally co-occurring with the cyanotoxin. Long-term exposures to MCY-LR induce dramatic changes in root development of *P. australis* (Máthé et al., 2007, 2009). Root elongation is inhibited, radial expansion of primary root cells occurs instead. Lateral root primordia are formed abundantly at the vicinity of primary root tips (Máthé et al., 2009). Radial expansion of shoot tip cells is induced by MCY-LR in another aquatic plant, *Ceratophyllum demersum* under laboratory conditions (Szigeti et al., 2010). Similar shoot alteration occurred in *C. submersum* naturally co-existing with a MCY containing cyanobacterial bloom in a small Hungarian pond (Ujvárosi et al., 2019). This study shows that even relatively short-term cyanotoxin treatments induce inhibition of root elongation and promote initiation of lateral root development in Arabidopsis-related to altered levels of PINs and in consequence, auxins. Thus, the model presented here suggests that developmental alterations induced by the cyanotoxin in natural plant

communities originate from alterations of local auxin concentrations in roots.

To conclude, MCY-LR induces alterations of root development in the model plant Arabidopsis and this is correlated to changes in the levels of PIN auxin efflux carriers and of local auxin levels in roots. These changes are related directly to the protein phosphatase inhibitory effects of the cyanotoxin at 24 h treatments. The present study is the first one to show such phytotoxic effects of a cyanotoxin. It contributes to a better understanding of phytotoxicity of a well-known cyanotoxin both under laboratory conditions and the real environment.

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## Availability of data and material

The authors declare that any experimental data for this manuscript are transparent and can be made visible at request.

## Declaration of competing interest

The authors declare they have no competing conflict of interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2021.130183>.

## Author contribution statement

C.F. performed experimental work and contributed to experimental design. C.M. participated in microscopy work and contributed to writing of manuscript. G.R., Á.C. and L.S. helped in performing work with CRK5:CRK5-GFP plants and *crk5* mutants. T.N. contributed to immunohistochemistry work and helped in experimental design. Z.K. and F.E. contributed with experimental design and work related to protein phosphatase activity assays. A.K. and E.P. helped in histochemistry work and assay of lateral root development. G.V. purified MCY-LR. T.G. participated in design and execution of experiments and contributed to writing of manuscript.

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