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Microcystin-LR induces chromatin alterations and modulates neutral single-strand-preferring nuclease activity in *Phragmites australis*

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ABSTRACT

Microcystin-LR (MCY-LR), a toxin produced mainly by freshwater cyanobacteria, is a potent inhibitor of type 1 and 2A protein phosphatases. As such, it induces biochemical, cellular and tissue alterations in vascular plants, including cell death. The aim of this study was the analysis of MCY-LR induced changes in the activity of single-strand preferring nuclease (SSP nuclease) isoenzymes that are possibly involved in programmed cell death (PCD) of *Phragmites australis* (common reed, an aquatic macrophyte) cells. We analyzed both single-stranded DNA (ssDNase) and double-stranded DNA (dsDNase) cleaving activities. Activity gels revealed a number of seven isoenzymes named bands A–G in control reed shoots and roots. Their activity was organ- and age-dependent. We stained nuclei of root tip meristematic cells and found total and marginal chromatin condensations at relatively short-term (2–10 days) cyanotoxin exposure. At 10–20 days of cyanotoxin treatment, the number of cells with condensed chromatin decreased, which coincided with the occurrence of necrotic cell death. In parallel, overall ssDNase activity increased in the short term (five days) and gradually decreased at 10–20 days of MCY-LR treatment. In this context, the most important changes occurred for isoenzyme G of 28–32 kDa in roots and isoenzyme F of 35–38 kDa in shoots. dsDNase activity of isoenzyme E was decreased by MCY-LR in shoots, but increased in roots at 10 days of exposure. We conclude that the early induction of chromatin condensation and increase of SSP nuclease activities is related to PCD that will lead to necrosis with the cease of all cellular activities, including a decrease in nuclease activity.

Introduction

It has long been known that single-strand-preferring nucleases (SSP nucleases) cleave primarily single-stranded DNA (ssDNA) and RNA. Based on the reactions they catalyze, SSP nucleases can be classified into endonucleases (their products are oligo- and/or mono-deoxynucleotides), exonucleases (their products are mononucleotides and require free 3′ or 5′ termini), and endo-exonucleases (Desai and Shankar, 2003). Well-characterized plant SSP nucleases include mung bean nuclease, *Avena* leaf nuclease, a tobacco culture nuclease (extracellular enzyme) and a senescence-induced bifunctional nuclease of *Arabidopsis*. Many plant SSP nucleases are type I nucleases (E.C.3.1.30.X) that are extracellular or intracellular heat-stable glycoproteins with endonucleolytic activity on ssDNA and RNA (Wilson, 1975; Farkas, 1982; Pérez-Amador et al., 2000). Type I nucleases include cereal enzymes co-localized with chromatin (Yupsanis et al., 1996). Their biological roles involve DNA replication, recombination and repair, or developmental processes such as seed germination and maturation (Desai and Shankar, 2003; Leśniewicz et al., 2010). Several ssDNase isoenzymes are induced during drought or oxidative stress, have an important functions in resistance to viral, bacterial or fungal pathogens as well as in the induction of senescence and programmed cell death (PCD), e.g., during xylogenesis (Wood et al., 1998; Pérez-Amador et al., 2000; Desai and Shankar, 2003; Leśniewicz et al., 2010).

Microcystin-LR (MCY-LR) is a well-known cyanobacterial toxin, frequently present in eutrophicated freshwaters (Carmichael, 1992). It is a potent inhibitor of type 1 and 2A protein phosphatases in eukaryotic organisms. Due to this property, the cyanotoxin interferes with a wide range of cellular processes, involving cell cycle regulation, signal transduction and the regulation of enzyme activities (MacKintosh and MacKintosh, 1994; Luan, 2003). With respect to vascular plants, a variety of toxin effects have been described. These include histological and cell cytoskeletal alterations (Máthé et al., 2007, 2009; Szigeti et al., 2010), interference with photosynthesis (Abe et al., 1996), the induction of oxidative stress and of corresponding defense enzyme activities (Pflugmacher, 2004).

Abbreviations: dsDNase activity, double-stranded DNA cleaving activity; MCY-LR, microcystin-LR; PCD, programmed cell death; ssDNase activity, single-stranded DNA cleaving activity; SSP nucleases, single-strand-preferring nucleases.

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It was recently shown that another microcystin variant, MCY-RR, induces changes indicating the onset of PCD in tobacco BY-2 cells. These alterations include the induction of oxidative stress, chromatin condensation and changes in plasma membrane and mitochondrial organization (Yin et al., 2006; Huang et al., 2008).

Phragmites australis (Cav.) Trin. Ex Steud. (common reed) is a cosmopolitan freshwater and brackish water macrophyte important for serving as a habitat for epiphytic organisms and its capacity to accumulate heavy metals. It is used for remediation purposes (Ye et al., 1998). For such reasons, the study of the physiological changes induced by environmental factors is an important research topic in this plant (Armstrong and Armstrong, 2001). The effects of water eutrophication and heavy metals on histological structure or C and N metabolism have been intensively studied (Armstrong and Armstrong, 2001; Fediuc and Erdei, 2002; Mészáros et al., 2003). Freshwater eutrophication is known to cause the overproduction of toxic cyanobacterial biomass, and common reed often coexists with MCY-LR producing cyanobacteria (Yamasaki, 1993; Máthé et al., 2007).

Our previous studies showed that MCY-LR induces microtubule disorganization and cell death/necrosis in tissue-cultured *P. australis* plantlets (Máthé et al., 2007, 2009). Therefore, in an attempt to understand the cellular and biochemical mechanisms of PCD in a MCY-LR treated aquatic macrophyte, we aimed to look for chromatin changes and the associated nuclease activities, known to be involved in the initiation of cell death. Many authors have found that stress-inducible nucleases often have a neutral or near-neutral pH optimum. Neutral nucleases are involved in the early stages of plant PCD, such as during the death of megagametophyte cells of white spruce (He and Kermode, 2003). Therefore, neutral pH conditions are widely used for the detection of SSP nuclease isoenzymes on activity gels (for an example, see Wood et al., 1998; Yupsanis et al., 1996; M-Hamvas et al., 2003). Thus, after examining pH optima of SSP nucleases on activity gels, we chose pH 6.8 as an optimum for the detection of *P. australis* nucleases. In this study, we show for the first time that MCY-LR induced PCD is associated with changes of SSP nuclease activities in plant cells.

Materials and methods

The purification of MCY-LR

Microcystin-LR (MCY-LR) was purified from *Microcystis aeruginosa* BGSD 243. The purification method has been described previously (Kós et al., 1995). In brief, cyanotoxin was extracted from cells with 5% (v/v) acetic acid, purified on a DE-52 column (Whatman) and desalted on Waters Sep-Pak® cartridges. The purity of MCY-LR was ≥90% as checked by HPLC and capillary electrophoresis methods (Vasas et al., 2004).

Plant material and cyanotoxin treatments

Phragmites australis (common reed) plantlets were regenerated from stem nodal embryogenic calli (Máthé et al., 2000). Cyanotoxin treatments were performed essentially as described previously (Máthé et al., 2007, 2009). Plantlets of 60 ± 3.2 (SE) mm height with well-developed shoot and root systems were used for the start of MCY-LR treatments. The cyanotoxin was added to 2 mL of liquid modified MS medium (Murashige and Skoog, 1962) in a concentration range of 0.5–40 μg mL^{-1} (0.5–40.2 μM). Toxin exposure times were 2–20 days.

The study of chromatin organization

Reed roots were fixed with 4% (v/v) formaldehyde in phosphate buffered saline (PBS), then treated with 40% (w/v) sucrose (Reanal, Budapest, Hungary) dissolved in PBS and cryosectioned with a Leica Jung Histoslide 2000 microtome (Leica, Nussloch, Germany). 10–15 μM thick sections were stained for chromatin; sections were washed with PBS and then cells were permeabilized by 10 min treatment with PBS containing 0.5% (v/v) Triton X-100 (Reanal, Budapest, Hungary). After three washes for 5 min, sections were stained for 40 min with 3 μg mL^{-1} of 4′,6′-diamidino-2-phenylindole (DAPI, Fluka, Buchs, Switzerland). Sections washed with PBS were mounted on microscopic slides in a drop of antifading buffer containing 0.1% (w/v) *p*-phenylenediamine (Sigma–Aldrich, Budapest, Hungary) in 90% (v/v) glycerol (Reanal, Budapest, Hungary), with pH adjusted to 8.0. The occurrence of chromatin condensation in root tip meristematic cells was examined with an Olympus Provis AX-70/A fluorescence microscope equipped with an Olympus Camedia 4040 digital camera (Olympus, Tokyo, Japan). Nuclear DNA was observed with the aid of a 320–360 nm excitation filter. Experiments concerning chromatin condensation were repeated three times and representative structures are presented in Results section.

Nuclease activity gels

The detection of single-strand preferring nuclease (SSP nuclease) activities on protein gels was performed by following the principles of Blank and McKeon (1989), Wood et al. (1998) and M-Hamvas et al. (2003). Cell-free protein extracts were prepared from shoots and roots in a buffer containing 10 mM Tris–HCl (Sigma–Aldrich, Budapest, Hungary), pH 8.0, 150 mM NaCl (Reanal, Budapest, Hungary) and 14.6 mM 2-mercaptoethanol (Sigma–Aldrich). In order to maintain nuclease activities, samples were not boiled prior to electrophoresis. The protein content of extracts was assayed by the method of Bradford (1976). 13 μg protein was loaded onto each well of 10% SDS-polyacrylamide gels containing 10 μg mL^{-1} heat denatured (for ssDNase activities) or intact chicken blood DNA (for dsDNase activities). Gel electrophoresis was performed according to Laemmli (1970). Reed shoot and root samples were loaded together with a molecular weight marker (Sigma–Aldrich, Budapest, Hungary). After electrophoresis, enzymes were renatured for 30 min in water, then by three washes in a buffer containing 10 mM Tris–HCl, pH 6.8 and 20% (v/v) 2-propanol, followed by washing in Tris–HCl, pH 6.8. For the detection of SSP nuclease isoenzymes, gels were incubated (generally for 24 h, 39 °C) in Tris–HCl, pH 6.8. After incubation, gels were stained with 0.5 μg mL^{-1} ethidium bromide (Sigma–Aldrich, Budapest, Hungary). Nuclease isoenzymes were detected as clear, unstained bands, due to DNA hydrolysis. Their molecular weight was estimated with the aid of UVI-TEC® software. Activities of isoenzymes were quantified with the CpAtlas® software that calculates the number of square pixels of a given protein band. Measurements were repeated at least six times, and the results are presented as mean ± SE values calculated with the Sigma Plot 10.0® software.

All experiments concerning the analysis of SSP nuclease isoenzymes were performed at least three times, and representative results are presented.

Results

The effect of MCY-LR on chromatin organization in P. australis meristematic cells

MCY-LR induced changes of chromatin organization in both primary and lateral root tip meristems of *P. australis* (Fig. 1). At two days of exposure of primary roots, 20–40 μg mL^{-1} MCY-LR induced nuclear shrinkage with total chromatin condensation as well as marginal chromatin condensation. If exposure times were longer (5–20 days), such alterations appeared at lower cyanotoxin con-