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In vivo influence of cyanobacterial toxins on enzyme activity and gene expression of protein phosphatases in Alfalfa (*Medicago sativa*)

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ABSTRACT

Irrigation of crop plants with surface water can be a threat if cyanobacterial toxins are present in the water. Cyanotoxins are known to cause adverse effects in plants. Microcystin (MC), a cyclic heptapeptide, with more than 70 structural variants, is a frequently occurring toxin. MC is a specific inhibitor of serine/threonine protein phosphatases 1 and 2A (PP1 and 2A), important regulatory enzymes in eukaryotic cells. Protein phosphatases consist of a catalytic subunit and one or more regulatory subunits. In Alfalfa several isoforms of the catalytic subunit of PP1 (*Ms*PP1α, *Ms*PP1β, *Ms*PP1γ, *Ms*PP1δ, *Ms*PP1ε) and PP2A (*Ms*PP2A Cα/β/γ) are known along with isoforms of the regulatory subunits of PP2A (*Ms*PP2A Aα/β, *Ms*PP2A Bα/β). The *in vivo* effect of environmentally relevant concentrations of cyanobacterial components on the mRNA transcript level of the subunits of protein phosphatases 1 and 2A in Alfalfa (*Medicago sativa*) was examined using semi-quantitative RT-PCR. Plants were exposed for one week to 5 μg L^{-1} microcystin-LR, microcystin-LW, okadaic acid and to cell-free cyanobacterial crude extracts from *Microcystis aeruginosa* containing 5 μg L^{-1} microcystin-LR and a toxin-free crude extract from *Synechocystis* spp. The protein phosphatase activity *in vivo* was inhibited when exposed to toxins and crude extract containing microcystin-LR, no change was induced by *Synechocystis* crude extract. The gene expression of the *Ms*PP1γ subunit and the *Ms*PP1ε subunit was induced in plants exposed to MC-LW.

1. Introduction

Cyanobacterial blooms can spread under certain environmental conditions (elevated nutrient level, temperature and sunlight). Many cyanobacterial species are known to produce different types of toxins. Microcystins (MC) are one of the most common cyanotoxins. These cyclic heptapeptides occur with more than 70 structural variants (Zurawell et al., 2005). Cyanobacteria have been associated with toxic effects on wildlife and domesticated animals (Galey et al., 1987). Cyanotoxins are a major health concern in areas of the world which exhibit conditions optimal for the

Abbreviations: MC-LR, microcystin-LR; MC-LW, microcystin-LW; OA, okadaic acid; *Mic*-CE, *Microcystis aeruginosa* crude extract; *Syn*-CE, *Synechocystis* spp. crude extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PP1 and 2A, protein phosphatases 1 and 2A; *Ms*PP1α/β/γ/δ/ε, isoforms of catalytic subunit of PP1 in *Medicago sativa*; *Ms*PP2A Aα/β, *Ms*PP2A Bα/β, isoforms of regulatory subunit of PP2A in *M. sativa*; *Ms*PP2A Cα/β/γ, isoforms of catalytic subunit of PP2A in *M. sativa*.

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growth and occurrence of cyanobacterial blooms. Due to the widely used spray irrigation technique using surface water which might be contaminated with cyanobacteria, a transfer of cyanobacterial toxins into crop plants is possible.

It has been shown that plant seedlings can take up cyanotoxins (Peuthert et al., 2007) causing inhibitory effects on development, root growth and photosynthesis (Smith et al., 1994; Abe et al., 1996; McElhiney et al., 2001; Pflugmacher et al., 2006).

A number of naturally produced toxins including microcystins are known to be potent inhibitors of serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) which belong to the major phosphatases in eukaryotic cells that dephosphorylate serine and threonine residues (MacKintosh et al., 1990; Cohen, 1989). They play a key role in the regulation of plant physiology and development (Luan et al., 2001). Biochemical and genetic studies in plants implicate PP1 and/or PP2A activity in signal transduction, hormonal regulation, mitosis, and control of carbon and nitrogen metabolism (Smith and Walker, 1996).

The combination of a catalytic subunit with different regulatory subunits converts PP1 and 2A into many different forms with distinct substrate specificities, restricted sub-cellular locations and diverse regulation. In Alfalfa five isoforms of the catalytic subunits of PP1 (*Ms*PP1α, *Ms*PP1β, *Ms*PP1γ, *Ms*PP1δ, *Ms*PP1ε) have been characterized (Pay et al., 1994; Vissi et al., 1998). The *Ms*PP1 isoforms exhibit different steady-state mRNA levels in different plant organs suggesting tissue-specific function (Vissi et al., 1998). Furthermore, a number of regulatory subunits of PP2A (*Ms*PP2A Aα/β, *Ms*PP2A Bα/β, *Ms*PP2A Cα/β/γ) have been characterized (Pay et al., 1994; Tóth et al., 2000) in Alfalfa. One of the B subunits was induced by abscisic acid and specific A–B subunit combinations exhibited parallel organ-specific expressions. Tóth et al. (2000) suggested that the structurally similar isoforms may have different functions and that some of the heterotrimer structures are preferred in a given plant organ.

The mechanism by which microcystin inhibits PP1 has been studied in detail (Goldberg et al., 1995; Holmes et al., 2002). Microcystin interacts with three distinct regions on the surface of the catalytic subunit of protein phosphatases: the metal binding site, the hydrophobic groove and the C-terminal groove and thus blocks access to the active centre of the enzyme (Holmes et al., 2002).

Adda, the hydrophobic side chain of MC, fits into the hydrophobic groove at the active site. The carboxyl group of the glutamic acid and the adjacent carbonyl oxygen of MC form hydrogen bonds with two water molecules at the metal binding site. The carboxyl group of the methyl aspartic acid of MC interacts with Arg-96 and Tyr-134 of PP1. The L-Leu side chain of MC fits alongside a tyrosine at the edge of the C-terminal groove near the active site. A covalent linkage between the methyl dehydroalanine side chain of the toxin and Cys-273 of PP1 was observed in the crystal structure (Goldberg et al., 1995). By inhibiting protein phosphatases the balance with protein kinases is disrupted and a hyperphosphorylated state in cytosolic and cytoskeletal proteins is produced (Toivola et al., 1994).

The aim of this study was to investigate *in vivo* the effects of several cyanotoxins and cyanobacterial crude extracts on gene expression of isoforms of PP1 and PP2A and on the activity of protein phosphatase 1 in *Medicago sativa*.

2. Materials and methods

2.1. Toxins and cyanobacterial crude extracts

The cyanobacterial material of *Microcystis* (MZ13 culture) was obtained from Prof. V. Vasconcelos (CIIMAR, Portugal). To prepare cell-free crude extract (*Mic*-CE), 20 g dry weight of MZ13 culture was suspended in 500 mL Milli-Q water (Waters, Eschborn, Germany) and stirred on ice for 15 min. After ultrasonication on ice, centrifugation of the resulting slurry was done at 22,000*g* for 15 min. Supernatant was collected and stored on ice. The pellet was reprocessed in the same manner as described above five times and the extracts were combined thereafter. The extract was stored in the deep freezer (−80 °C) before use. Toxin analysis of the crude extract revealed the presence of microcystin-LR (MC-LR) in a concentration of 168 μg mL^{-1}. The extract was diluted with water to a final concentration of 5 μg L^{-1} MC-LR used for the experiments. A second crude extract (*Syn*-CE) was prepared in the same manner using a non-toxic *Synechocystis* spp. (PCC6803) culture from our institute. Analysis of this crude extract confirmed no microcystins as expected.

Purified, commercially available toxins: microcystin-LR (MC-LR), microcystin-LW (MC-LW), okadaic acid (OA) were obtained from Axxora (Grünberg, Germany) and diluted to a final concentration of 5 μg L^{-1} toxin with water.

2.2. Determination of microcystins

Analyses were performed as described in Pflugmacher et al. (2006) using a Waters HPLC system (Waters, Eschborn, Germany) with photodiode array detector (waters 2996) detection. Separation was carried out on a Symmetry 5 μm C18 column (3.9 × 150 mm). The mobile phase consisted of solvent A: Milli-Q water and solvent B: acetonitrile (Rathburn, Walkerburn, UK) both containing 0.1% (v/v) trifluoracetic acid. Solvent B was linearly increased from 30% to 45% over 10 min at a flow rate of 1 mL min^{-1}. Column temperature was maintained at 40 °C and the injection volume was set to 80 μl.

2.3. Plant material and exposure experiments

Seeds of the Alfalfa variant Europe were purchased from Greenfield (Krefeld, Germany). They were germinated and separately cultivated in batches with garden soil for two months under semi-field conditions in a field outside the Leibniz Institute of Freshwater Ecology and Inland Fisheries until September 2006. The exposure was carried out for one week. Each plant was watered on the soil with 4 mL of water containing purified toxins or the two different crude extracts every two days. The toxin concentration used was 5 μg L^{-1}, except the *Synechocystis* containing no toxin. To obtain similar conditions for the crude extract experiments, chlorophyll levels of *Syn*-CE and *Mic*-CE were determined at a wavelength of 625 nm. The *Syn*-CE was than diluted to the chlorophyll concentration of the *Mic*-CE used for