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Chromium-induced physiological and proteomic alterations in roots of *Miscanthus sinensis*

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ABSTRACT

Despite the widespread occurrence of chromium toxicity, its molecular mechanism is poorly documented in plants compared to other heavy metals. To investigate the molecular mechanisms that regulate the response of *Miscanthus sinensis* roots to elevated level of chromium, seedlings were grown for 4 weeks and exposed to potassium dichromate for 3 days. Physiological, biochemical and proteomic changes in roots were investigated. Lipid peroxidation and H_2O_2 content in roots were significantly increased. Protein profiles analyzed by two-dimensional gel electrophoresis revealed that 36 protein spots were differentially expressed in chromium-treated root samples. Of these, 13 protein spots were up-regulated, 21 protein spots were down-regulated and 2 spots were newly induced. These differentially displayed proteins were identified by MALDI-TOF and MALDI-TOF/TOF mass spectrometry. The identified proteins included known heavy metal-inducible proteins such as carbohydrate and nitrogen metabolism, molecular chaperone proteins and novel proteins such as inositol monophosphatase, nitrate reductase, adenine phosphoribosyl transferase, formate dehydrogenase and a putative dihydrolipoamide dehydrogenase that were not known previously as chromium-responsive. Taken together, these results suggest that Cr toxicity is linked to heavy metal tolerance and senescence pathways, and associated with altered vacuole sequestration, nitrogen metabolism and lipid peroxidation in *Miscanthus* roots.

1. Introduction

Heavy metal contamination is a cause of major environmental hazards worldwide, leading to losses in agricultural yields and harmfully affecting human health when contaminants enter the food chain. Chromium (Cr) is the seventh most abundant element on earth. It exists in nature in both trivalent (Cr III) and hexavalent (Cr VI) forms, of which the latter is more toxic [1]. Cr compounds cause environmental pollution as a result of a large number of industrial operations, including mining, pigment manufacturing, petroleum refining, leather tanning, wood preserving, textile manufacturing, pulp processing and fungicide development [2]. In India, about 2000–3200 tones of elemental Cr leak to the environment annually with a Cr concentration ranging between 2000 and 5000 mg L^{-1} [3]. Very high level of Cr(VI) contamination (14,600 mg kg^{-1} in ground water and 25,900 mg kg^{-1} in soil) has been reported in some sites of Oregon state, USA [4]. Generally, most Cr (VI) added to soil is promptly reduced to the inert form Cr (III) by several agents. However, re-oxidation of Cr (III) to Cr (VI) occur by microorganisms and, therefore, both states should be regarded hazardous for the environment and for humans [5]. Both forms cause serious damage to plant tissues and organs at differing concentrations. Cr phytotoxicity can result in the inhibition of seed germination, pigment degradation, disturbances in the nutrient balance and the generation of reactive oxygen species (ROS), which induces oxidative stress and alterations in antioxidant enzyme activities [6]. In the cell, free system reactivity of Cr is generally considered by its interaction with glutathione (GSH), NADH and H_2O_2-generating hydroxyl radicals (OH^-) [7]. Both Cr III and VI react with cellular H_2O_2, generating highly reactive hydroxyl radicals.

Industrial chromium wastes are generally treated with physicochemical processes before they are released into the environment. Following primary treatments, the methods of removal of residual chromium (polishing) are expensive and the efforts are often insufficient [8]. Consequently, residual Cr are released to the environment and accumulated in agricultural products through water,

Abbreviations: 2-DE, two-dimensional gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PMF, peptide mass fingerprinting; ROS, reactive oxygen species; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TBARS, thiobarbituric acid reactive substance; V-ATPase, vacuolar-type H^+-ATPase; UDP-GlcDH, UDP-glucose dehydrogenase.

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air and polluted soils [6]. Soils from numerous sites in the USA are contaminated with Cr at levels ranging from 1 to 1500 mg Cr kg^{-1} [9]. Soil pollution generates extra costs for soil management and pollution control. The uses of plants for soil phytoremediation by means of degradation (phytodegradation), adsorption (rhizofiltration) and absorption (phytoextraction) are efficient, renewable, and natural processes that are leading competitors in the search for solutions to these contamination issues. Unfortunately, most known hyperaccumulator plants have very low biomass and/or slow growth rates, are difficult to cultivate on a commercial scale and have very few commercial uses. Therefore, attention has been focused on several biomass crops that have fast growth rates and high biomass and are able to accumulate moderate to large amounts of heavy metals without sacrificing biomass gain. Most studies involving Cr overaccumulation have focused on extreme examples, representing plants native to highly Cr-rich environments [10,11]. Very little attention has been paid to commercially important biomass-producing crops. *Miscanthus sinensis*, a perennial rhizomatous C4 grass, is a potentially efficient, sustainable carbon-neutral producer of lignocellulosic biomass, making it very suitable and promising for the production of biofuels and fiber [12]. *Miscanthus* sequesters higher amount of Cr to the aerial part at extremely toxic levels, whereas the overall ability of this species to remove Cr from the solution is higher at moderate toxicities [13]. These suggest that *M. sinensis* is a potential bioaccumulator of Cr and other heavy meals.

Heavy metal-accumulating plants have expansive, advanced antioxidant defense systems and other important features that enable them to acquire tolerance [14,15]. Unfortunately, little is known about the molecular basis of excess heavy metal tolerance. Unlike other heavy metals, such as As, Cu, Pb and Cd, the partitioning of Cr by phytochelatin synthesis has not been observed; therefore, the detoxification mechanism for this metal is poorly understood [6]. Molecular events underlying Cr toxicity and the defense-related signal transduction process have been only partially elucidated. A number of genes potentially involved in Cr tolerance and accumulation were assessed by cDNA-AFLP and reported [16]. Recently, the combination of genome-wide transcriptome profiling and metabolome analysis has been reported in Cr-stressed rice plant [17].

Proteomics, the comprehensive and quantitative analysis of proteins that are expressed in a given organ, tissue or cell line, provides unique insights into biological systems that cannot be acquired from genomic or transcriptomic approaches. Proteomics has been used extensively to investigate the protein expression pattern under abiotic stresses. Expression pattern of maize proteins in response to high concentrations of Cr (340–1019 μM) have been described for the first time by [18]. However, no proteomic study has been carried on *M. sinensis* in response to Cr stress. Therefore, we carried out a proteomic analysis of *M. sinensis* roots subjected to Cr stress to identify proteins or primary targets, hoping to gain a more thorough understanding of the molecular basis of heavy metal tolerance in this species.

2. Materials and methods

2.1. Plant growth and treatments

M. sinensis (cv. Kosung) seeds were planted on commercial potting mix in plastic trays and allowed to germinate in a growth chamber. Three weeks after germination, the seedlings were transferred to hydroponic cultures supplied with half strength Hoagland nutrient solution (H2395, Sigma, USA). pH of the medium was adjusted to 5.8. To ensure proper growth, the solutions were aerated with aquarium aerators. Following a 1-week hydroponic adaptation, the seedlings were subjected to treatments of 0, 50, 100, 200, 300, 500, 750 and 1000 μM potassium dichromate ($K_2Cr_2O_7$). After a 3-day treatment, the roots were excised from untreated (control) and treated seedlings and used for proteomic and physiological analyses. The entire experiment was conducted under light conditions (500 μmol m^{-2} s^{-1}, 16/8 h light/dark period) at 25 °C and 65% humidity.

2.2. Determination of Cr accumulation in roots

After 3 days of treatment, root samples were washed five times with deionized water to remove surface Cr salts. The samples were dried in an incubator at 60 °C for 72 h, weighed, and then ground to a fine powder. Approximately 1 g of fine powder from each treatment group was digested, using a ternary solution ($HNO_3/H_2SO_4/HClO_4$, 10:1:4 v/v), and the total Cr in the digestion solution was determined with a graphite furnace atomic absorption spectrophotometer (GFAAS) (PerkinElmer SIMAA 6000, Norwalk, CT, USA) [19]. Three different biological replicate root samples were used for the analysis.

2.3. Measurement of lipid peroxidation and hydrogen peroxide

Lipid peroxidation was estimated by measuring the concentrations of 2-thiobarbituric acid-reactive substances (TBARS) as described previously [20]. Briefly, 300 mg of powdered tissue were homogenized in 20% trichloroacetic acid (TCA), containing 0.5% 2-thiobarbituric acid, and heated at 95 °C for 30 min [21]. The TBARS concentrations were measured as the malondialdehyde (MDA; $\varepsilon = 155\ mM^{-1}\ cm^{-1}$) concentrations, which were determined at A_{532} and corrected for nonspecific turbidity at A_{600}. The hydrogen peroxide (H_2O_2) concentrations were measured spectrophotometrically as described by [22]. Briefly, H_2O_2 was extracted by homogenizing 300 mg of tissue samples with 3 mL of phosphate buffer (50 mM, pH 6.8), containing the catalase inhibitor hydroxylamine (1 mM). The homogenate was centrifuged at 6000 × *g* for 25 min. A mixture comprised of 3 mL of extracted solution and 1 mL of 0.1% titanium sulfate in 20% (v/v) H_2SO_4 was centrifuged at 6000 × *g* for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm. The H_2O_2 level was calculated, using the extinction coefficient 0.28 $\mu mol^{-1}\ cm^{-1}$.

2.4. Protein extraction and 2-D electrophoresis

Proteins were extracted from the root sample using a phenol extraction method according to our previous paper [23]. Briefly, 750 mg of tissue was homogenized with a Mg/NP-40 extraction buffer [0.5 M Tris–HCl, pH 8.3, 2% (v/v) NP-40, 20 mM $MgCl_2$, 1 mM phenyl methyl sulfonyl fluoride, 2% (v/v) β-mercaptoethanol and 1% (w/v) polyvinyl polypyrrolidone] and fractionated with water-saturated phenol, followed by centrifugation at 12,000 × *g* for 15 min. The proteins were recovered from the supernatant by precipitation with ammonium acetate in methanol. The protein samples were then quantified using the Lowry method [24] and subjected to two-dimensional gel electrophoresis (2-DE) using a standard procedure. The protein samples were dissolved in a reswelling buffer [8 M urea, 1% CHAPS, 0.5% (v/v) IPG buffer pH 4–7, 20 mM dithiothreitol (DTT), and a trace of bromophenol blue]. A total of 500 μg of dissolved protein sample was applied to the immobilized pH gradient (IPG) dry strip (pH 4–7, 18 cm) for 13–14 h, followed by focusing for 47,500 V-h using an IPGphor (Amersham Bioscience, Uppsala, Sweden). After isoelectric focusing (IEF), the IPG strips were equilibrated for 15 min in an equilibration buffer [50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a trace of bromophenol blue] containing 10 mg/mL DTT, followed by 15 min in an equilibration buffer