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Arsenate-induced expression of a class III chitinase in the dwarf sunflower *Helianthus annuus*

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Abstract

Dwarf sunflower cultivars of *Helianthus annuus* were exposed to 30 mg/L As alone and in combination with other toxic metals (30 mg/L each of Cd, Cr, and Ni). Total uptake of metals at the end of a 17-day exposure cycle showed variable sequestration of the metals in roots, leaves, and stems with metal concentrations highest in roots and lowest in leaves. The presence of other metals appeared to have little effect on the uptake of As. Proteins were extracted from leaves of plants exposed to arsenic alone or in combination with other metals and size-fractionated by one-dimensional SDS-polyacrylamide gel electrophoresis. Several polypeptides, with molecular masses ranging from less than 10 to over 45 kDa appeared to be unique in extracts obtained from leaves of plants in response to arsenic stress. One polypeptide was induced strongly. It had an approximate molecular mass of 32 kDa and was recovered from gels and analyzed using LC–MS/MS. It was identified as a class III chitinase, whose gene appeared to be induced at the transcription level when As was present.
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Keywords: Arsenic stress; Chitinase; *Helianthus annuus*; Hyperaccumulator; Phytoremediation

1. Introduction

Arsenic contamination is widespread as the result of industrial applications ranging from its use as pesticides, mining and smelting of heavy metals and burning of high arsenic coals (Smith et al., 1998). The resulting contamination of water and soil poses a major health problem since As is toxic to most living systems including humans, animals, and plants (Fowler, 1983). The health problems caused by arsenic are quite severe in some parts of the world, including the Guizhou province of China, Mexico and Bangladesh (Alkorta et al., 2004). Remediation of arsenic-contaminated soils and water has, therefore, become a major environmental issue. Phytoremediation is regarded as a promising approach for heavy metals (Terry and Banuelos, 2000; Pilon-Smits, 2005). Over 400 plants have now been reported as hyperaccumulators of one or more metals (Baker et al., 2000). For example, *Thlaspi caerulescens* is capable of accumulating Zn and Cd in its leaves (Reeves and Brook, 1983). *Ipomea alpine* (Baker and Walker, 1990), *Haumaniastrum robertii* (Brooks, 1977) and *Sebertia acuminate* (Jaffre et al., 1976) hyperaccumulate Cu, Co, and Ni, respectively. Recently, Cong and Ma (2002) discovered that Ladder brake (*Pteris vittata* L.) is able to hyperaccumulate arsenic. It accumulates as much as 2.3% of arsenic when grown in an arsenic-amended soil (Ma et al., 2001).

The underlying mechanisms of hyperaccumulation of metals are beginning to be understood. In general, the hyperaccumulation of toxic metals is influenced by the efficiency and capacity of uptake, intercellular transportation and sequestration (Yang et al., 2005). Some of the principles governing these processes could be attributed to the plant's ability to mobilize metals in its rhizosphere (Krishnamurti et al., 1997), induce various ligands to distribute and sequester metals (Grille et al., 1985) or increase its antioxidative capacity (Schützendübel and Polle, 2002). A number of plants have been shown to increase synthesis of phytochelatins (PCs) by phytochelatin synthase (PCS) in the cytosol to combat Cd stress (Grille et al., 1985; Heiss et al., 2003). The involvement of PCs in metal accumulation was further demonstrated in that the *Arabidopsis* gene encoding PCS conferred a marked increase in metal tolerance, since a mutant

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in PCS made the plants sensitive to Cd and arsenate (Ha et al., 1999). Furthermore, PC formation could also be induced when *Arabidopsis* was exposed to other metal ions, as well as arsenite and arsenate (Rauser, 1995). Metallothioneins (MTs) are another group of low molecular weight cysteine-rich peptides that are important in Cd-detoxification in animals (Hammer, 1986) and are considered as potential ligands in resisting metal toxicity in plants. However, their role in metal tolerance in plants remains unclear. On the other hand, metallothionein mRNA could be induced in *Arabidopsis* seedlings by Cu (Zhou and Goldsbrough, 1994). Heavy metal hyperaccumulation could also be related to the ability of plants to induce proteins which are not directly involved in binding to metals. Heat shock proteins, for instance, show increased expression in response to stress conditions including heavy metals (Lewis et al., 1999). These proteins are known to be involved in normal protein folding but could also be involved in the repair of membrane proteins damaged by metals (Lewis et al., 2001). Similarly, chitinases are often expressed as the result of biotic stress such as fungal and bacterial infection, but might have other functions such as the response to abiotic stress by heavy metals (Graham and Sticklen, 1994; Passarinho and de Vries, 2002). Chitinases are a group of enzymes that catalyze the degradation of chitin, a linear β1-4-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc) and are found in fungi, yeasts, animals as well as in plants; they are divided in two families (18 and 19), and subdivided in five classes.

As an initial approach to using the dwarf sunflower, *Helianthus annuus,* for phytoremediation of metal containing soil the phytoremediation efficiency was investigated when the sunflowers were grown hydroponically and then exposed to various heavy metals, alone or in combination.

2. Materials and methods

2.1. Contaminant source

Sunflowers were exposed to heavy metals in solutions containing single metals (As, Cd, Cr or Ni) and combinations of Cd plus Cr and Ni, with or without As. Each heavy metal was added at 30 mg/L. The 30 mg/L refers to the concentration of the individual metal, not the compound added. The metals were applied as As^{5-} ($Na_2HAsO_4{\cdot}7H_2O$), Cd^{2+} ($CdSO_4{\cdot}8H_2O$), Cr^{3+} ($CrCl_3{\cdot}6H_2O$), and Ni^{2+} ($NiSO_4{\cdot}6H_2O$).

2.2. Cultivar source and determination of metals in plants

Experiments were conducted with two different *Helianthus annuus* cultivars (Sundance and Teddy Bear). Seeds of each cultivar were grown hydroponically in Rockwool, a non-reactive, nonabsorbent support system and sustained with a standard hydroponic nutrient solution (Cropking Corp., Seville, OH). The seeds were grown in a greenhouse illuminated with natural light. The average temperature of the greenhouse was 28 °C (winter) and 35 °C (summer) during the day and 20 °C at night. After a 4 week growth period, seven sunflowers were transferred to a PVC trough. One set of plants was then subjected to a 4 L solution containing 30 mg/L of the metals as described above. Solutions were introduced at a complete recycle rate of 6 gallons per hour. After 17 days of exposure, the plants were harvested and the experiment repeated with the Teddy Bear cultivar, using fresh solutions.

To harvest the plants, the Rockwool was first carefully removed from the roots. Roots, leaves and stems were then sectioned and weighed. Half of the collected tissue were shipped to be immediately used (i.e., not dried) for protein and RNA isolation (Cleveland State University). The remaining tissue was dried followed by an acid digestion for metal analysis using flame atomic absorption spectroscopy (FAAS) (University of Akron; Buck 200 AA).

2.3. Analytical methods

To determine heavy metal content the procedure proposed by Zheljazkov and Erickson (1996) was used. One gram of milled plant tissue was soaked in 20 mL of concentrated nitric acid for 6 h. Next, the mixture was concentrated by boiling to 50% of the original volume and then 4 mL of perchloric acid was added and the mixture was refluxed for 90 min. The solution was then diluted with water to a final 20 mL and analyzed by FAAS. The detection limits for the target metals were 0.028 mg/L for Cd, 0.078 mg/L for Cr, 0.14 mg/L for Ni and 0.25 mg/L for As. The concentrations of Cd, Cr, Ni, and As were determined by calibration curves obtained using standards solutions of pure metal ions (Fisher Scientific, Waltham, MA).

2.4. Statistical analysis

The results from the four studies were analyzed using MINITAB version 14 software. Experimental results were compared using a general linear model. Statistical significance was determined using Tukey comparisons with P-values <0.05 were considered statistically significant.

2.5. Protein extraction and in-gel tryptic digestion

The plant tissues were mashed with mortar and pestle in liquid nitrogen, and 100–200 mg were placed in extraction buffer. The plant extraction solution Focus™ Plant Proteome (G-Bioscience, St. Louis, MO) was used following the manufacturer's instructions, then centrifuged for 20 min at 15,000 × g. The supernatants were collected and the protein concentrations were measured using the Bradford method with albumin as the reference protein solution. SDS-PAGE (Weber and Osborn, 1969) was performed to establish distribution of proteins, molecular mass of the proteins present in the control and the metal-exposed plant biomass. Gels were stained with Coomassie Brilliant Blue.

Proteins from stained gels were cut out with a razor blade and treated with the in-gel tryptic digestion kit (Pierce, Rockford, IL) following the manufacturer's instructions. Digestion was conducted overnight at 30 °C.